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A MICRO-RESPIROMETER FOR SINGLE PREPUPAE OF *DROSOPHILA MELANOGASTER* MEIGEN

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(*Received* 22 *March* 1945)

(With Two Text-figures)

INTRODUCTION

Many workers have measured the rate of oxygen consumption of puparia of *Drosophila* (see Ellenby, 1945); all have worked with groups of animals. As the rate of oxygen consumption is proportional to the surface area and not to the first power of the body weight (Ellenby, 1945), the mean weight of a group of animals is an incorrect basis for the comparison of oxygen consumption. Nor is the mean weight raised to some power strictly correct, for the size composition of groups of animals will vary, and the mean weight to some power will not be equal to the mean of the individual weights to the same power. It therefore seemed desirable to investigate the oxygen consumption of individual animals. Moreover, for a number of reasons, information was required on the oxygen consumption of triploid prepupae; such animals can only be distinguished in the imaginal stage, so that it is necessary to use individuals and to establish their constitution subsequently. An apparatus suitable for such work is here described; it was found very convenient in use and it was thought it might be of interest to workers in related fields.

APPARATUS

Essentially, the apparatus consists of a battery of micro-respirometers of the type used by Gerard & Hartline (1934) for the measurement of the oxygen consumption of nerve. In the original design, a capillary tube, which contains the nerve and a strip of filter paper soaked in KOH, is sealed at one end with vaseline, and contained in a large jacket-tube; the movement of a drop of manometric fluid in the capillary is measured with a microscope. The apparatus is of the differential type, the two vessels being the capillary tube and the jacket-tube; but the volume of the latter is very large in comparison with that of the former; in these circumstances the pressure change in the jacket-tube during an experiment is negligible, and the movement of the manometric fluid indicates the change of volume. The apparatus is therefore simple to use, and calibration merely involves the determination of the volume per cm. of the capillary tube; already it has been shown that it can be readily adapted to different purposes (Waddington, Needham & Brachet, 1936; Brachet & Shapiro, 1937). Figs. 1 and 2 show the present design. Each respirometer consists of a capillary tube (*A*), about 0·5 mm. internal diameter and 9 cm. long, joined to another capillary of about the same length but wider bore (*B*), the end of which is ground to take a small cup (*C*). The prepupa (*D*) is placed just inside the wide capillary, and filter paper (*E*), cut with a paper punch and moistened with KOH, is carried at the bottom of the cup. A drop of oil is introduced at the open end and its position measured from the most convenient of a number of marks etched on the fine capillary. The prepupa is removed from the apparatus with a hooked platinum wire, the animal being pulled on to a small piece of folded card from which it is easily transferred to the pan of a torsion balance.

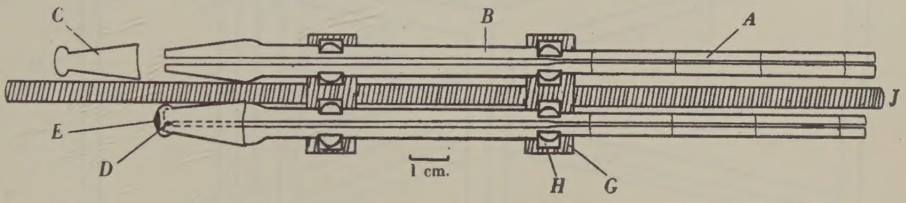

Fig. 1.

The large capillary projects into the potash cup. This brings the prepupa close to the CO_2 absorbing surface and also reduces the effective volume of the cup; and the vaseline on the ground glass of the capillary provides an effective barrier to the creeping of potash to the experimental animal.

Two such capillary assemblies are contained in a single jacket-tube. The capillaries are mounted on ebonite blocks (*G*) by means of metal clips (*H*), the two blocks connected by an ebonite rod (*J*), and the whole is so constructed that it fits firmly into the jacket-tube. All metal parts are coated with vaseline. In use the capillaries are seldom removed from their mountings, even for cleaning.

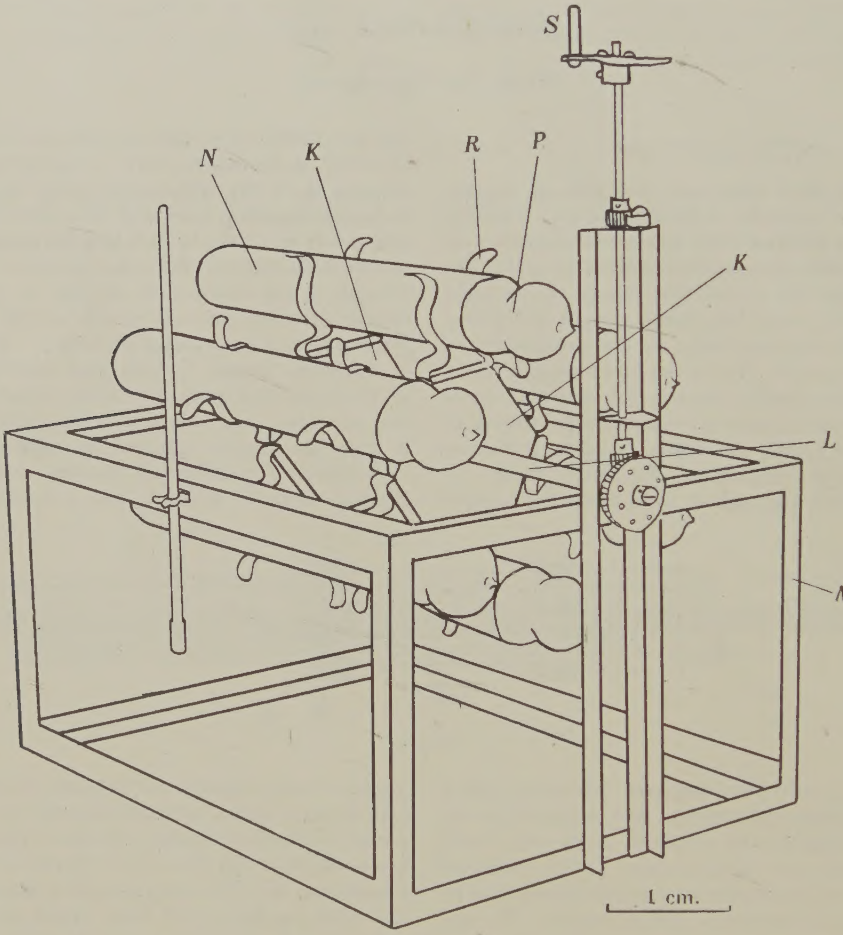

Fig. 2.

The complete apparatus is shown in Fig. 2. Two hexagonal ebonite blocks (*K*) are mounted on a horizontal spindle (*L*), carried by a framework (*M*) and connecting by a cogwheel with a vertical shaft. Six jacket-tubes (*N*), with hollow ground-glass stoppers (*P*), each containing two capillary assemblies, are carried on the hexagons by metal clips (*R*). The whole is immersed in a water-bath. Turning the handle (*S*) rotates the horizontal spindle and thus brings each of the capillaries, in turn, under the field of a low-power microscope mounted on a traversing screw; the oxygen consumption of twelve separate prepupae can thus be determined simultaneously.

As designed, the apparatus is not among the more sensitive of micro-respirometers; but it suits the range for which it was designed very well. The volume of the fine-bore capillary is about 15 cu.mm., and larger prepupae almost consume this volume of oxygen in the 5 hr. during which they were studied. The average excursion was about 1 cm. an hour, so that if a scale were mounted with the capillaries, accurate readings could be taken directly with a microscope, and the traversing screw dispensed with. While the fine capillary increases the sensitivity, the wider capillary and potash cup increase the total volume of air to about 0·5 c.c. and thus ensure that the oxygen consumption does not fall to any significant extent during an experiment. Excluding the air displaced by the contained apparatus, the volume of the jacket-tube is about 250 c.c.; the volume ratio of jacket- and capillary tubes, while not as high as in the original apparatus, is therefore still sufficiently high for the pressure changes in the former to be ignored.

Oxygen consumption falls very rapidly during the first few hours of the prepupal period (Ellenby, 1938), and it is therefore very desirable to reduce the equilibration period to a minimum. In the present case, the apparatus was used in a constant temperature room and the equilibration period thus eliminated; but for other work, in which the rate of oxygen consumption is not changing so rapidly with time, simple modification would permit the apparatus to be used under more ordinary circumstances.

SUMMARY

An apparatus is described, of the Gerard-Hartline type, suitable for the measurement of the oxygen consumption of single pupae of *Drosophila*. The design is such that the oxygen consumption of twelve separate animals can be conveniently measured simultaneously.

The apparatus was constructed in 1938 in the Department of Zoology, University College, London. I am very grateful for the encouragement I received from Prof. D. M. S. Watson, F.R.S., and for the co-operation of Mr J. R. Redpath, the departmental mechanic.

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THE REACTIONS OF ISOLATED PARTS OF *SPIROSTOMUM*

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(*Received* 17 *July* 1945)

(With One Text-figure)

The determination of the reactions and behaviour of isolated fragments of a protozoan is of value to the study of three problems, namely, the relationship between the nucleus and behaviour, the detection of any area in the organism specialized for the reception of external stimuli, and in the case of a ciliate, the role played in normal swimming by the cilia of different regions of the body. Recently, an account has been given of the reactions of fragments of *Amoeba proteus* (Clark, 1944). The present paper is an extension of the work to include a ciliated form.

The behaviour of ciliate fragments was first studied by Balbiani (1889) and Verworn (1901). Neither author gives a detailed account of the work, but they agree that, in general, the fragments move and behave in much the same way as the entire organisms. Jennings & Jamieson (1902) examined the effects of chemical and mechanical stimuli on anterior and posterior fragments of *Stylonichia*, *Oxytricha*, *Stentor*, *Spirostomum* and *Paramecium*. They concluded that: 'As any portion of a crystal is organized like the entire crystal, so in the Ciliata any piece of the body, apparently, is organized so as to move and react to stimuli in the same manner as does the entire animal.'

Alverdes (1922, 1923) believes that in *Paramecium* the reception of thermal and chemical stimuli is confined to the extreme anterior end, and that *Spirostomum* and *Stentor* show sensitivity to thermal stimuli only at the anterior end but to chemical stimuli over the entire body surface. More recently, Horton (1935) determined the sensitivity of anterior and posterior halves of *Paramecium* to weak-acid stimulation but failed to confirm Alverdes's claim that only anterior fragments are responsive to chemical stimulation. He has also observed that, contrary to earlier accounts, posterior halves are more sensitive to acid than anterior halves, and attributes this to the greater permeability to acid of the newly formed pellicle at the anterior surface of the posterior fragment, as compared with the permeability of the undamaged pellicle at the front end of the anterior fragment. In connexion with the relative importance of regional cilia for swimming, he found that fragments lacking the oral cilia swim spirally like intact animals, indicating that the spiral track traced out by *Paramecium* '...is not dependent on the shape or on the action of the oral groove cilia, but is produced by the co-ordinated activity of the body cilia'.

Two strains (S and SS) have been used in the present work, corresponding to the *major* and *minor* varieties of *Spirostomum ambiguum* (see Bishop, 1923). 'Wheat' cultures were used, to which two rat faecal pellets were added to every 30 c.c. of medium. Specht (1935) and Seyd (1937) have both reported favourably on the addition of mammalian faeces to cultures of *Spirostomum*.

To designate the levels at which cuts were made and localized stimuli applied, points were chosen at quarter-length intervals (see Fig. 1). Points 1 and 5 were the extreme anterior and posterior ends. Point 3 was the estimated middle of the body. The mouth generally occupied a position half way between points 3 and 4. Point 2 was estimated solely by comparison with points 1 and 3, there being at this level no morphological structure to which reference can be made. For further details on the morphology, reference should be made to the papers of Bishop (1923, 1928).

THE RESPONSES OF WHOLE ANIMALS TO GENERAL AND LOCALIZED MECHANICAL STIMULI

If an intact *Spirostomum* is subjected to a non-localized stimulus, e.g. by tapping gently against the edge of the container, the organism contracts strongly, darts back a little and then gradually relaxes, revolving on its long axis in a clockwise direction (facing the anterior end), and maintaining the reversal in the effective beat of the cilia for several seconds. When relaxation is complete, the anterior end swings out towards the aboral side, and forward swimming is resumed. If the stimulus is weak, temporary reversal may occur without any contraction of the myonemes.

Localized mechanical stimuli, applied by means of a glass needle, were separated by irregular time intervals of from one to five minutes and in different animals were applied according to different systems. In any one experiment, ten stimuli were localized at each of the points from 1 to 5, giving a total of 50. In some cases they were applied systematically, in a numerical sequence, i.e. ten at point 1, ten at point 2, ... and so to point 5. In others, they were again applied systematically but in reversed order, commencing with ten at point 5, ten at point 4, until point 1 was reached. Finally, in a third series, the localization followed no regular system at all, each point receiving a total of about ten stimuli administered at random, according to which point was most conveniently situated with regard to the tip of the needle at the instant of stimulation.

A total of thirty individuals was examined in this manner and yielded quite consistent results. Generally, stimuli applied at points 1, 2 and 3 result in contraction and temporary ciliary reversal, while stimuli localized at points 4 and 5 produce contraction accompanied by swimming forwards. In a very small number of cases, as Jennings (1899*a*) has noted, a stimulus at the anterior end produces only contraction, the animal remaining stationary for a few moments while relaxation of the myonemes is taking place. The important point is that, in the vast majority of cases, the organism definitely responds in relation to the localization of the stimulus. The response consists essentially of movement away from the source of the stimulus. Both Jennings (1899*a*) and Alverdes (1923) have noticed the greater sensitivity of the anterior end, which appears to have a lower threshold than the posterior end to a variety of stimuli. This feature, characteristic of many other ciliates, will be returned to when considering the reactions of anterior and posterior fragments.

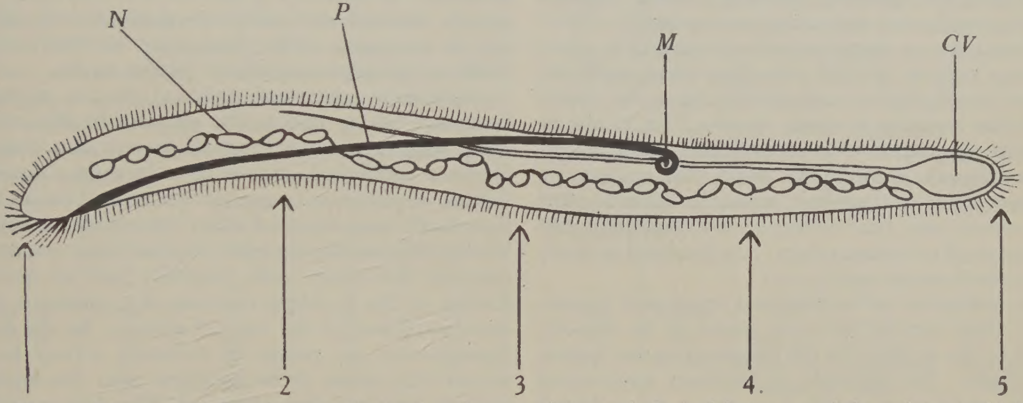

Fig. 1. Diagram of *S. ambiguum*, illustrating the essential morphological features and the points at which localized stimuli were applied and transections carried out. *CV*=contractile vacuole; *M*=mouth; *N*=macronucleus; *P*=peristome.

THE RESPONSES OF FRAGMENTS TO MECHANICAL STIMULI

While swimming forward, the intact organism revolves slowly on its long axis in an anticlockwise direction, with the result that the anterior end traces out a narrow spiral. If a temporary ciliary reversal, or a contraction followed by forward movement takes place, rotation is still anticlockwise, but if contraction and ciliary reversal both take place (e.g. as a result of a general stimulus, or one localized at points 1, 2 or 3) during the subsequent backward movement and relaxation, the rotation is clockwise. Precisely the same points hold for fragments. Rotation on the long axis is anticlockwise for all cases except that of contraction, followed by swimming backwards, when it is clockwise.

On contact with the glass thread, the organism contracts strongly. After the transection has been made, the anterior portion reverses and moves backwards in a wide spiral for a quarter of a minute or more. If the fragment is very small the path traced out is so compact a spiral as to be almost circular. On the other hand, larger fragments tend to move more in a straight line, revolving on the long axis the while. Owing to the slight curvature of the body, the posterior end of the fragment traces out a narrow spiral. Within a minute or so, the ciliary beat again reverses and the fragment swims forward in a manner similar to that of an intact individual, differing only in that the tendency to follow a spiral course is more conspicuous.

The posterior fragment, immediately after transection, usually remains inactive for a few seconds, then suddenly shoots backwards for some distance, revolving only very slowly on the long axis. There is a slight tendency towards a spiral course, but this is certainly not so obvious as in the case of anterior fragments. Nor is the rate of movement so great in the posterior pieces and the reversal often lasts for a few seconds only. It is usually followed by a period of inactivity of about a minute's duration before forward swimming is resumed. In a small number of cases, the posterior fragment showed no ciliary reversal at all, simply moving forwards very slowly after transection. The chief difference, then, between anterior and posterior fragments immediately after cutting is the greater activity of the former and the tendency of the latter to remain inactive for a few seconds before either continuing forwards, or reversing. Inactivity on the part of the anterior fragment is usually of only two or three seconds' duration.

Unfortunately, no earlier workers have indicated the age of the fragments used in their studies. Consequently, this point must be borne in mind when considering the discrepancies between their findings and my own, for, to anticipate the conclusion of this paper, fragments immediately after isolation from the whole do not show normal behaviour. On the other hand, regulation of form is accompanied by a return to normal in the behaviour and the responses to stimuli.

Jennings & Jamieson (1902) state that both anterior and posterior halves move and react in the same way as the whole individual, revolving anticlockwise when swimming and, when stimulated (general or localized?), backing and turning to the aboral side, the anterior fragment turning through a greater angle and swimming more rapidly. They also found that a posterior half or one-third, when subjected only to a mild stimulus, often oscillates back and forth, but reverses and turns to the aboral side if the stimulus is more severe. This is not in complete agreement with one of Jennings's earlier papers (1899*a*), in which he states that a posterior fragment reacts as the whole animal but fails to turn to the aboral side, from which he has concluded that 'the power of initiating a turn...is localized in some way in the anterior end'.

The behaviour of a fragment examined immediately after section has been found to be directly related to the position of the fragment in the animal as a whole. For example, a localized mechanical stimulus applied at points 1, 2 and 3 in an intact individual is followed by contraction and ciliary reversal. Therefore, a fragment taken from any region between points 1 and 3, or even the entire anterior half, responds to all stimuli by contracting and swimming backwards, regardless of whether the stimulus is localized at any point or is general. A stimulus localized at points 4 or 5 in the intact animal is followed by contraction with increased rate of swimming forwards. Precisely the same reaction is obtained from fragments taken from the posterior quarter of the whole organism, again regardless of the localization of the stimulus. A striking experiment is to bisect a *Spirostomum*, to allow the two halves to recover from the shock and to resume swimming forwards, and then to tap sharply against the edge of the watch glass. Provided the tap is severe enough, both fragments will contract, but while the anterior half darts backwards, the posterior swims forwards. They are responding in relation to their positions in the organism as a whole.

After a period of from 12 to 48 hr. has elapsed, the fragments show complete morphological regulation and, coinciding with this, commence to behave as whole animals, showing differential responses towards localized stimuli and darting backwards when stimulated generally. Thus a statement on the normality or abnormality of the behaviour of a fragment is incomplete if there is no accompanying specification of the age of the fragment.

From the results of observations on responses to localized stimuli in more than thirty-five pairs of fragments, the general conclusion was drawn that the regulation of behaviour proceeds in both anterior and posterior fragments which are at least one-eighth the length of the original organism. In four experiments the fragments were less than a tenth of the original length, and all failed to show the reappearance of differential responses within 72 hr.

Fragments taken from the middle region of the body between points 2 and 4 are interesting in that they generally fail to show any locomotory response at all for several hours after section. Such fragments move very slowly and, when stimulated either generally or at some point on the body surface, merely contract and remain motionless until relaxation is complete, when they swim forwards again. Differential responses appear within 24 hr.

Jennings's contention (1899*a*) that a posterior fragment fails to turn to the aboral side after swimming backwards away from the source of a stimulus has been confirmed, and seems to be quite a constant feature of posterior fragments for several hours after they have been isolated from the whole organism. When differential responses appear, however, a turn towards the aboral side becomes just as much a feature of the avoiding reaction of a posterior fragment as of that of the normal animal. In the intact *Spirostomum* the power of initiating a turn to the aboral side seems to be localized near the anterior end, as Jennings has observed. The localization is not at the extreme anterior end, however, for cutting off the tip of the organism, at about a quarter of the distance between points 1 and 2, does not affect the ability of the hinder portion to turn to the aboral side. It is concluded that the localization is in the anterior quarter of the body but is not at the extreme tip.

The peculiar oscillation that Jennings observed when a posterior fragment is stimulated is also a common, but not constant, feature of recently isolated merozoa. It is most readily observed in posterior fragments resulting from section between points 2 and 3. When stimulated at its anterior end, such a fragment may contract and dart back a little, only to swim forwards a short distance and then swim backwards again. This may be repeated several times before normal swimming is resumed in the original direction.

Reference has already been made to the difference in the sensitivity of the anterior and posterior ends of certain ciliates, and which is described in some detail by Jennings (1906). In an attempt to obtain quantitative evidence for this difference the following experiments were carried out.

Fragments were mounted in a moist chamber in such a way that, by means of an electromagnetic hammer striking against the chamber, they could be subjected to repeated general stimuli of constant intensity. The results of several experiments are grouped in Table 1, which gives the percentage of contractions produced in each individual by thirty constant intensity shocks spaced at 5 sec. intervals. The figures are given for the whole animal and for the corresponding anterior and posterior fragments immediately after section.

These figures illustrate a general feature, that the larger the fragment, the more sensitive it is, regardless of whether it is from the anterior or posterior regions of the whole animal. Hence, a large posterior fragment may appear to be more sensitive than a small anterior one (e.g. in Exps. SS 12 and 13). On the other hand, if the fragments are of nearly equal size, the anterior half definitely responds more readily than the posterior. Over a period of 36 to 48 hr., all the fragments show a gradual return to the sensitivity of the original organism.

Table 1. *Responses to constant intensity shocks. Table of the percentage of contractions elicited from whole animals, and their resulting anterior and posterior fragments, by several mechanical stimuli of constant intensity. Each was stimulated thirty times, with a time interval of 5 sec. between successive stimuli*

No. of exp.	Level of section	Whole organism	Anterior fragment	Posterior fragment
		%	%	%
SS 13	1½	85	45	70
SS 12	2	95	40	85
SS 19	3	88	70	45
SS 11	3	85	60	10
SS 22	4	90	80	40
SS 16	4	70	75	25
SS 15	4½	85	95	0

In a second series of experiments, a determination was made of the minimal mechanical shock required to produce ten successive contractions, the time interval between the stimuli in each group being 30 sec. Typical results are presented in Table 2. Figures for the fragments were determined immediately after transection. Variation in the intensity of the mechanical shock was obtained by dropping small weights from a known height.

From these figures it may again be concluded that large fragments respond more readily than small, and that anterior fragments respond more readily than posterior ones. The threshold rises rapidly with decrease in the size of the fragment, but the rise is many times greater in posterior fragments, some of which often fail to respond regularly even towards extremely strong shocks (70,000–100,000 ergs). The threshold for all the fragments becomes smaller with the lapse of time, tending towards about 15,000 ergs, which was found to be the minimal mechanical shock that would regularly produce contraction in a whole individual. This figure, of course, holds only for infrequent stimulation and presupposes no complications due to habituation.

It seems certain then, that there is a marked difference in the sensitivities of the anterior and posterior ends of *Spirostomum*. It will be remembered that Horton (1935) found that posterior halves of *Paramecium* are more sensitive to acid stimulation than anterior halves, and he explained this by assuming that the newly formed pellicle covering the cut surface is more permeable than that of the rest of the body. As the posterior fragment moves forwards, the first part of it to encounter a chemical stimulus is the newly formed pellicle, whereas, the first part of an anterior fragment to encounter the chemical is the original, undamaged pellicle. Hence, any increase in the permeability of the pellicle would afford a ready explanation of apparently increased sensitivity on the part of the posterior fragment. Thus, Horton's observation does not necessarily conflict in any fundamental way with the current, widely held opinion that greater sensitivity is characteristic of the anterior end of ciliates. The following observations on the effects of cyanide solutions on fragments of *Spirostomum* are relevant.

Table 2. *Table of the minimum, general mechanical shock found necessary to produce contraction of each individual on ten occasions, the stimuli being separated from each other by 30 sec. In the case of the posterior fragment of SS 35, a shock of 73,500 ergs produced a maximum of only 60% of contractions. The minimum shock required to produce ten successive contractions (i.e. 100%) must have been considerably greater than this and could not be determined without the risk of damaging the apparatus*

No. of exp.	Level of section	Intensity of shock. Producing 10 successive contractions		
		Whole organism ergs	Anterior fragment ergs	Posterior fragment ergs
SS 28	2	15,680	31,360	23,520
SS 2	3	14,700	17,600	29,400
SS 21	3	14,700	15,680	31,360
SS 17	3	14,700	14,700	58,800
SS 35	4	15,680	14,700	73,500 (60%)

It has been suggested by Pantin (1930), and demonstrated by Clark (1942), that the cytolytic action of a strong solution of potassium cyanide on amoebae is due to the action of the high concentration of potassium ions in a solution of high alkalinity. *Spirostomum* disintegrates readily in *M*/100 KCN, and whether the cytolysis is due to alkalinity alone or to alkalinity plus potassium ions, the effect is essentially a surface one. If there is any difference between the permeabilities of the old and newly formed pellicles, immersion in cyanide should reveal it.

The individual to be examined was placed in a few drops of $M/100$ KCN contained on a depression slide, and covered quickly with a cover slip in order to prevent the rapid loss of HCN vapour from the solution. The time for cytolysis to occur was measured with a stop watch. The mean time for 50 intact spirostoma was found to be 270 sec., ranging from 46 to 367. With only one exception, the disintegration commenced at some point within the anterior third of the organism and proceeded caudally, illustrating a well-defined susceptibility gradient, which has already been described by Child (1924). Groups of anterior and posterior fragments were immersed in cyanide at varying intervals after transection, and in Table 4 the mean times for cytolysis are given. In all cases the organisms were cut at level 3, giving fragments of nearly equal size.

Table 3. *Table showing the relation between the mean time of cytolysis of fragments in* $M/100$ KCN *and the time elapsing since isolation*

Time after bisection hr.	Total number of fragments examined	Mean time for cytolysis sec.	
		Anterior	Posterior
0	20	18	16
1	20	150	35
12	10	277	257
24	10	294	305

The mean time for the cytolysis of a whole individual is 270 sec.

It will be seen that, following bisection, there is a very marked increase in the cyanide sensitivity, both anterior and posterior halves disintegrating rapidly. It is highly significant that, in every case, cytolysis commenced at the recently repaired surfaces, with the result that the anterior fragments disintegrated from their posterior ends. One hour after bisection there is a rise in the resistance of the fragments but cytolysis still commences at the newly formed surface, while 12 or 24 hr. after bisection, fragments resemble the whole animal both with regard to the order of magnitude of the time for cytolysis to occur, and to the appearance of an antero-posterior susceptibility gradient. These facts are most readily interpreted if we accept Horton's suggestion that the newly formed surface has a different permeability from the old.

THE RESPONSES TO A CHEMICAL STIMULUS

Using as a stimulus a dilute aqueous solution of sodium chloride, Alverdes (1923) found that both anterior and posterior fragments of *Spirostomum* show an avoiding reaction, demonstrating that the reception of a chemical stimulus is not confined to the anterior portion of the body. Blaettner (1925) briefly states that he confirms this observation. To reinvestigate the matter, localized stimuli were applied by means of a micropipette, using a dilute solution of neutral red (0·05%) in water as the stimulating agent. The use of a coloured solution is advantageous for it enables an estimation to be made of the degree of spreading of the stimulating agent from the point of application.

A second technique that has proved very valuable involves the use of a larger pipette with an orifice of about 0·1 mm. The ciliate is placed in a watch glass containing several drops of culture water and, when it is swimming in an approximately central position, the pipette is dipped beneath the surface of the water and a circle of dye solution is 'drawn' around the organism, which is thus enclosed within a small barrier. The diameter of the circle should vary from 2 to 10 mm., depending on the size of the organism. Soon the ciliate will reach the chemical barrier and, being stimulated at its anterior end, will move backwards. Provided the circle is of the correct diameter, this backward movement will bring the posterior end up to the dye of the opposite side of the circle and a response may again be observed. Thus the technique is a simple means of applying localized stimuli at the extreme ends of the animal. The experiment can be especially striking with organisms that move some distance backwards following a stimulus at the anterior end (e.g. *Spirostomum*, *Paramecium*, and *Frontonia*), but is naturally not suitable for organisms such as the hypotrichs, which move backwards only a short distance before turning to one side. Whole organisms and fragments were always examined individually, permitting a detailed observation of their reactions and movements. We will consider first the behaviour of whole animals, already described in some detail by Jennings (1899*a*, *b*) and Blaettner (1925).

On approaching the dye solution by the anterior end, *Spirostomum* hesitates, the tip may wave to and fro, as if testing the surrounding region, and then the ciliary beat is reversed. After moving back half a length, the animal swings out towards the aboral side through an angle of about 45°, and the direction of ciliary beat again changes and swimming forwards is resumed. In most cases the reaction is obtained before the anterior end actually reaches the coloured zone, indicating the detection of the chemical stimulus in a relatively high dilution. When enclosed within a circle of dye, *Spirostomum* may show this reaction as many as fifty times without contraction occurring. On the other hand, applying the dye locally at the anterior end by means of the fine pipette generally results in both ciliary reversal and contraction of the myonomes. Thus a weak chemical stimulus produces only ciliary reversal, while a strong stimulus elicits both reversal and contraction.

Enclosed within a circle of dye, the anterior end of the animal is repeatedly subjected to stimuli and, with each stimulus, there may be a gradual increase in the distance covered during the reversal of the ciliary beat. At the same time, the anterior end is turning through an appreciable angle after every reversal, and it is inevitable that sooner or later the posterior end of the animal will come in contact with the chemical barrier. No reaction is obtained unless the body definitely reaches the coloured zone, illustrating the relative insensitivity of the posterior end. When the posterior end does reach the dye, the intensity of the stimulus is relatively great and, as might be expected, contraction occurs and this is followed by a dart forwards.

As in the case of mechanical stimuli, the behaviour of fragments towards a chemical stimulus was found to be related to the degree of regulation that had occurred since transection was carried out. Fragments examined 24–48 hr. after isolation behave in exactly the same way as the whole animal. Consequently, the following account of aberrant behaviour concerns only fragments which had been isolated one or two hours before the experiments were made.

Anterior fragments less than three-quarters the length of the whole animal react to a chemical stimulus at either end by swimming backwards, contracting also if the stimulus is severe. If the stimulus is applied at the anterior surface, the reaction is fundamentally the same as that of the intact organism. A very small anterior fragment may show a slight difference in that, after reversing the direction of the ciliary beat, it may turn through two or three circles before swimming forwards again. Following a stimulus at the posterior surface, the reversal in the ciliary beat of course brings the fragment further into the dye. Contraction then occurs but the reversed swimming is maintained. Several more twitches may take place and if the dye is present in a very thick layer (as in the circle technique), streams of acid-reacting mucus are liberated from the body surface. Whether the fragment survives or not depends on the concentration of the dye and the thickness of the barrier that it constitutes.

A posterior fragment greater than about one-third the length of a whole animal shows differential responses to localized stimuli, but after the reception of a stimulus at the anterior surface it fails to turn to the aboral side, although normal ciliary reversal takes place. After moving back a short distance the fragment swims forward along the original course. Again encountering the chemical, it reverses a short distance and then once more moves forwards. It may oscillate back and forth in this manner for several seconds (see also Jennings, 1899*a*).

A small posterior fragment behaves in an extremely interesting manner when stimulated at its anterior surface. If the stimulus is mild, reversal and oscillation are observed. On the other hand, if the stimulus is severe, contraction of the myonemes occurs and the fragment shoots forwards into the dye.

THE RESPONSES TO HEAT

Alverdes (1923) claims that the perception of thermal stimuli in *Spirostomum* is confined to the anterior end of the body. The technical difficulties involved when attempting to localize a thermal stimulus are so great as to be almost insuperable, for conduction and convection rapidly render the stimulus diffuse. Fairly satisfactory results can be obtained using the methods described by Jennings (1904, 1906). In an intact individual a stimulus applied at the anterior end elicits the usual avoiding reaction, while one localized at the posterior end produces an acceleration in the rate of swimming forwards. The latter response is probably a physical effect on the rate of ciliary movement, for a very strong stimulus applied at the same point (4 or 5) produces contraction accompanied by a dart towards the source of the stimulus. There is thus a failure to show a true differential response to heat.

Fragments that have undergone regulation show the same reactions as the whole animal, while recently isolated merozoa show variations similar to those already described in the previous two sections. More than thirty pairs of fragments have been examined and in no case did the posterior portion fail to respond to a thermal stimulus. This is not in agreement with the findings of Alverdes (1923), but I can offer no explanation for the discrepancy.

DISCUSSION

The main point demonstrated in this work is that while no area of the body surface in *Spirostomum* is differentiated to form a special sense organ, there is a definite antero-posterior gradient in the behaviour pattern. Mechanical, chemical or thermal stimuli, provided they are of sufficient intensity to equal or exceed the threshold, will elicit responses when applied to any point on the body surface. These responses are generally made with reference to the localization of the stimulus, and are differential in nature, although this has not been demonstrated for the case of a thermal stimulus. I am unable to offer an explanation of why Alverdes (1923) found that only the anterior end is sensitive to heat. Even extreme posterior fragments never failed to give an avoiding reaction towards a mild thermal stimulus encountered at the anterior surface. The 'mechanical shock' experiments have demonstrated in a quantitative way the greater sensitivity of the anterior end over the posterior, a difference described on several occasions by many writers, principally Jennings and Child. That the fragments also swim similarly to whole individuals supports the view that the spiral track taken by a swimming ciliate is due to the co-ordinated activity of the body cilia, and not to the special action of the cilia of the mouth region. This is in general agreement with the views of Jennings & Jamieson (1902) and of Horton (1935), on the significance of the spiral track.

Jennings & Jamieson (1902) have expressed the opinion that a ciliate fragment behaves in the same way as the entire animal, and gave their 'crystal analogy' which was referred to at the beginning of this paper. They do not state what time elapsed between the isolation of the fragment and the application of the stimuli, and consequently criticism of their observations must be reserved, in so far as it will be valid only if they examined the fragments immediately after the animals had been bisected. If, however, several hours had elapsed, enabling the merozoa to regulate both morphological form and the nature of the responses, then the conclusion of these authors, that fragments respond like the whole organism, is correct. In the present paper, it is shown that, until regulation is complete, fragments behave with reference to the position they occupy as an integral part of the whole organism. In other words, *Spirostomum*, instead of being organized like a crystal, actually has a gradient pattern in its protoplasm. After isolation from the whole, any one part of the organism at first behaves as though it were still part of the pattern, but eventually a process of reorganization takes place, similar to the morphological reorganization that Dembowska (1925) and others have found during regeneration in the more highly organized hypotrichs. Regulation in the responses of a fragment to stimuli appears to be a further aspect of the tendency for an isolated portion of a morphogenetic field to reconstitute itself into a similar field of smaller size. Seyd (1937) studied the process of morphological regulation in fragments of *Spirostomum* and has also expressed the opinion that a definite regulatory agency, in the nature of a field, functions in the isolated merozoa.

It would appear that Jennings's suggestion (1899*a*), that the power to initiate a turn in *Spirostomum* is localized in the anterior end, is correct, for it has been consistently observed that posterior halves, when swimming back from the source of any stimulus, fail to turn towards the aboral side unless they have already completed the regulatory process. Although Bishop (1928) failed to find in the neuro-motor system of *Spirostomum* any anatomical structure which could be called a 'motorium' or controlling centre, it would be of some interest to trace the reorganization and rearrangement of fibres occurring during regulation.

SUMMARY

1. *Spirostomum* shows differential responses to localized mechanical and chemical stimuli.

2. The entire body surface is sensitive to mechanical, chemical and thermal stimuli.

3. The spiral path followed during swimming is due to the co-ordinated activity of the body cilia.

4. Immediately after isolation, fragments do not behave like normal animals, but respond with reference to the position they occupied in the whole organism.

5. When morphological regulation is complete, the responses of the fragments are normal.

6. As revealed by the study of the responses of fragments, *Spirostomum* is not organized like a crystal, but seems to have a definite gradient pattern.

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FERTILIZING CAPACITY OF SPERMATOZOA FOLLOWING COLD TREATMENT OF THE SCROTAL TESTES OF RABBITS

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(With Two Text-figures)

The fertility or sterility of a male depends on several factors, among which are the chemical constituents of the semen, the number, morphological integrity, motility, metabolism and viability of the spermatozoa. These factors, of course, are interrelated in effecting fertility or probability of fertilization.

Although in normal matings the number of spermatozoa in the ejaculate is much greater than the minimum required for fertilization, it was demonstrated by Walton (1927), and by Rowlands (1944), that fertility in the rabbit was reduced if the number of spermatozoa inseminated was less than 1 million, and that sterility occurred if the number of spermatozoa was less than 0·1 million. Therefore, in the practice of artificial insemination in animal husbandry, the importance to be attached to the number of spermatozoa used is stressed by all workers (Walton, 1933; Lambert & McKenzie, 1940).

In relation to fertility and sterility, the importance of sperm morphology, and the percentage of abnormal forms in the ejaculate have been emphasized by many workers (Williams & Savage (1925) and Lagerlöf (1936) on bulls; Gunn, Sanders & Granger (1942) on rams), and especially by human gynaecologists (Meaker, 1934; Moench, 1936; Lane-Roberts, Sharman, Walker & Wiesner, 1939). A percentage of abnormal spermatozoa in the semen higher than 20 is ordinarily considered liable to cause partial fertility or sterility. Data that support this statement are based upon clinical observation rather than on experiments.

Various types of abnormal spermatozoa have been described. Morphologically, one of the most conspicuous and distinctive types is that of tailless spermatozoa frequently present in the semen.

Since the writer (1943) found that application of ice to the scrotal testis or epididymis of a rabbit for 10 min. caused separation of the head and tail in about 50% of spermatozoa in the ejaculate, it was thought that this artificial production of abnormal spermatozoa might give a useful technique to investigate experimentally the relative importance of number of spermatozoa, and the proportion of abnormal sperms in relation to fertility or sterility. It would also be of interest to know the fertilizing capacity of the morphologically normal sperms which remained in the ejaculate, to determine whether they were also physiologically normal, and whether the sex ratio of the young produced by insemination of semen samples following cold treatment of the testis would be affected.

ANIMALS AND METHODS

Forty adult doe rabbits of mixed breeding were bought from the Cambridge market. They were fed on hay, concentrates with minerals and green grass or vegetables. They were weighed once a week, and were in very good condition during this study. Semen of four brother bucks from Dr Hammond's inbred 'L' strain was collected with an artificial vagina for insemination. The method of collection was similar to that described by Macirone & Walton (1938). The instrument used for insemination has been described by Walton (1930), and the method of insemination by Walton (1927). The does were mated with vasectomized bucks twice before insemination, or once before and once after, in order to induce ovulation. One ml. of saline sperm suspension, containing a known number of normal spermatozoa, and a known number of tailless sperm heads, was inseminated into the vagina of the does in all cases.

The scrotal testes or epididymides were treated with ice as described by Chang (1943) 1–10 days before the collection of semen. Ice was applied only once for 10 min. if 20–50% of sperm heads were required, and for 20–25 min. every day twice or three times if 70–95% of sperm heads were required for insemination.

The number of abnormal sperms other than the artificially produced tailless sperms, such as those with small head or with peculiar head, was counted. Since, however, the proportion of these was very small (less than 10%) and fairly constant in both treated and untreated samples, the number was not separately recorded, but is included under the same heading of 'sperm heads' throughout this paper.

Semen samples were first diluted with about 5 ml. of 0·9% NaCl. A small proportion of this suspension was then immediately diluted with a known volume of alkaline methylene-blue saline (Chang, 1943), and the number of normal sperms, sperm heads and other abnormal forms determined by counting with a haemocytometer. With this estimate of the cell constituents, the original saline suspension was further diluted to give a suspension containing the required number of normal sperms.

When the experimenter had prepared the sperm suspensions, an assistant mated the does with vasectomized bucks, and they were inseminated. The time interval between collection of semen and insemination was within 1 hr. (in most cases 20–40 min.), and the interval between sterile mating and insemination was a few minutes.

After insemination of a batch of does (usually 3–6) with 1 ml. of sperm suspension, the cell constituents were then counted again from three separate preparations, and the average recorded.

The motility of the spermatozoa was examined on a warm stage before and after insemination. Motility can be classified as either progressively motile, actively motile, or sluggishly motile. Only the proportion of progressively motile sperms was taken into consideration. Within the limits of accuracy of observation, the proportion of progressively motile spermatozoa was not affected by dilution, and, in fact, coincided very closely with the proportion of normal sperms. For these reasons, therefore, the observations on motility have not been included in the published data.

Smears of the saline suspensions were made on slides and stained with acid-fuchsin or with haematoxylin, and the sperm morphology of normal and treated samples examined.

A series of parallel inseminations was carried out with semen from the same bucks before ice treatment of the testes, or 17 days after, and these inseminations served as normal controls.

Pregnancy of the doe rabbits was diagnosed by palpation of the abdomen and mammary glands two weeks after insemination. Nest-making of pseudo-pregnant rabbits was recorded.

The young born were killed at birth, and the sex determined by examining the external genitalia, and this diagnosis checked by examination of the gonads. If a doe died during pregnancy, the number of foetuses was counted, but if very young, the sex of the foetus could not be determined. The does were inseminated again 5–7 days after taking away the young.

This work was started in February 1944, and continued until January 1945, but stopped for 2 months, from the middle of August to the middle of October, when the fertility of the stock rabbits is fairly low.

RESULTS

Altogether, 111 inseminations were made with semen from treated animals.* Table 1 summarizes the results.

Table 1. *Inseminations with semen from males after treatment of the scrotal testes with cold*

Total no. of normal sperms millions	% of tailless sperm heads and abnormals	No. of does	% of does fertile	Sex of young M.	F.	?	Ratio males per 100 females	Mean litter size per insemination	Mean litter size per pregnancy
Range 51·5–21·0 Mean = 32·9	Range 17–62 Mean = 34·7	15	80	50	40	0	125	Range 4–10 Mean = 6	7·5
Range 19·3–14·3 Mean = 16·8	Range 58–73 Mean = 67	13	76·9	40	33	7	121	Range 4–10 Mean = 6·2	8·0
Range 10·5–5·5 Mean = 7·4	Range 71–91 Mean = 79	16	87·5	56	59	0	95	Range 5–11 Mean = 7·9	9·0
Range 4·2–3 Mean = 3·3	Range 91–95 Mean = 93·4	14	92·8	43	49	0	88	Range 3–12 Mean = 6·6	7·1
Range 2·7–2·4 Mean = 2·5	Range 90–95 Mean = 92	17	76·5	18	21	30	86	Range 1–9 Mean = 4·1	5·3
Range 2·0–1·2 Mean = 1·6	Range 90–98 Mean = 94	19	57·8	21	26	0	81	Range 1–11 Mean = 2·5	4·3
Range 0·8–0·4 Mean = 0·6	Range 96–97 Mean = 96·6	17	0	—	—	—	—	0	0

The complete data were first arranged in order of decreasing number of normal spermatozoa used in each insemination, and then divided into groups of about seventeen does so as to give a statistically adequate number in each group. So far as possible, the total number of spermatozoa used in each insemination was kept constant, and only the percentage of sperm heads varied. In seventy-four inseminations the total number lay between 30 and 70 millions. With this number it proved difficult to reduce the number of normal spermatozoa below the minimum required for full fertility even after severe treatment of the testes. In a subsequent thirty-seven inseminations the total number of sperms inseminated was reduced to between 15 and 30 millions. There was no evidence that this reduction in total number either increased or decreased the chances of the normal spermatozoa effecting fertilization.

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* Limitations of space prevent publication of the full data, but these may be obtained from the Librarian, School of Agriculture, Cambridge.

The fertilizing capacity of the sperm suspension is indicated in Table 1 by the percentage of fertile inseminations in each group and by the average number of young produced per insemination. Fig. 1 shows the results of the whole experiment graphically, and the following conclusions can be drawn: (1) Provided the number of normal sperms present in the suspension is not reduced below 3 millions, the fertilizing capacity of the suspensions, as shown by the percentage of fertile does and by the average number of young born, is unaffected, although more than 90 % of tailless sperm heads may be present in the suspension. (2) When the number of normal sperms decreases from 3 to 1 million, partial fertility occurs. The reduction in the average number of young born is more rapid than the reduction in the percentage of fertile does. (3) A minimal number of normal sperms is required for full fertility. A decrease in the number will cause partial fertility and eventually sterility, but an increase above the minimum will not increase fertility. (4) When the number of normal sperms is below 1 million, sterility occurs. (5) It is evident that separated sperm heads have no fertilizing capacity, since when the number of normal sperms was less than 3 millions, partial fertility and sterility occurred, although there was a high number of sperm heads present.

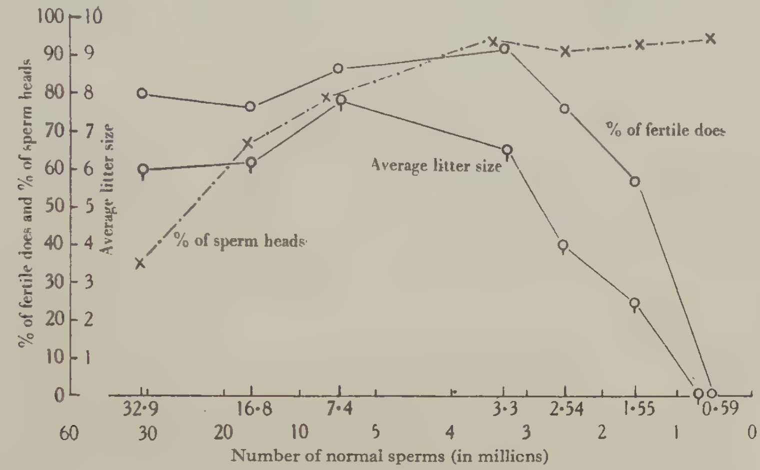

Fig. 1. Fertilizing capacity of spermatozoa following low temperature treatment of scrotal testes.

Table 2. *Insemination with semen from untreated males*

Total no. of normal sperms millions	% of sperm heads and abnormals	No. of does	% of does fertile	Sex of young				Mean litter size per insemination	Mean litter size per pregnancy
				M.	F.	?	Ratio males per 100 females		
Range 63·5–47·5 Mean = 55·8	Range 2–12 Mean = 7·6	14	78·6	45	27	6	166	Range 2–10 Mean = 5·6	7·1
Range 37·0–23·4 Mean = 29·5	Range 10–16 Mean = 14	14	92·9	55	62	0	89	Range 6–12 Mean = 8·4	9·0
Range 11·1–10·2 Mean = 10·6	Range 16–26 Mean = 20	9	100·0	31	43	0	72	Range 6–12 Mean = 8·2	8·2
Range 3·8–2·7 Mean = 3·3	Range 5–12 Mean = 9	12	75·0	23	39	0	59	Range 4–10 Mean = 5·2	6·9
Range 1·65–1·23 Mean = 1·41	Range 13–14 Mean = 13·6	9	77·8	6	5	46	—	Range 5–13 Mean = 6·3	8·1
Range 0·42–0·33 Mean = 0·38	Range 8·6–8·8 Mean = 8·7	11	73	29	35	0	83	Range 4–11 Mean = 6·8	8·0

Table 2 summarizes the results of sixty-nine control inseminations with semen from untreated males. The data have been set out in the same way as the experimental material, and both sets of data are compared together in Fig. 2. It will be seen that the fertilizing capacity of the untreated spermatozoa remains high, even when their number has been reduced to less than 0·4 million, whereas with an even higher number of treated spermatozoa, fertilizing capacity has fallen to zero. This clearly indicates that a high proportion of what appeared to be morphologically normal spermatozoa in the treated samples was in fact incapable of fertilization. Approximately ten times the number of apparently normal sperm in the treated samples were required to maintain full fertility, and hence only about 10% were both morphologically and physiologically normal. Preparations stained with either acid-fuchsin or haematoxylin failed to reveal any difference between the morphologically normal sperms in either the experimental or control samples.

The distribution of abnormal sperms other than separated sperm heads, such as sperms with small, or misformed heads, was the same in both treated and untreated samples, whether the count was made in the haemocytometer after dilution with alkaline methylene-blue saline, or when examined in smears stained with acid-fuchsin or haematoxylin.

Sex ratio. According to the genetic theory of sex determination, equal numbers of male- and female-determining spermatozoa are produced. It has been advanced that in certain cases of disturbed ratio, the sex ratio at conception is not unity and that the deviation may be due to a differential mortality of spermatozoa prior to fertilization. After analysis of the data obtained from experiments on the survival of spermatozoa *in vitro*, Walton (1930) concluded that the sex ratio is not significantly altered by keeping the sperms outside the body. His results did not, therefore, support the theory of a differential mortality of male- and female-determining spermatozoa.

The data shown in Table 1 indicate a slight but consistent decline in the ratio of male births with reduction in the total number of morphologically normal spermatozoa inseminated; but since many factors may be involved, such as the severity of treatment, and the time of storage of the spermatozoa in the epididymis, a statistical analysis on such few numbers would clearly not be profitable (cf. Walton, 1938).

Efficiency of artificial insemination. It is of interest to compare the efficiency of artificial insemination

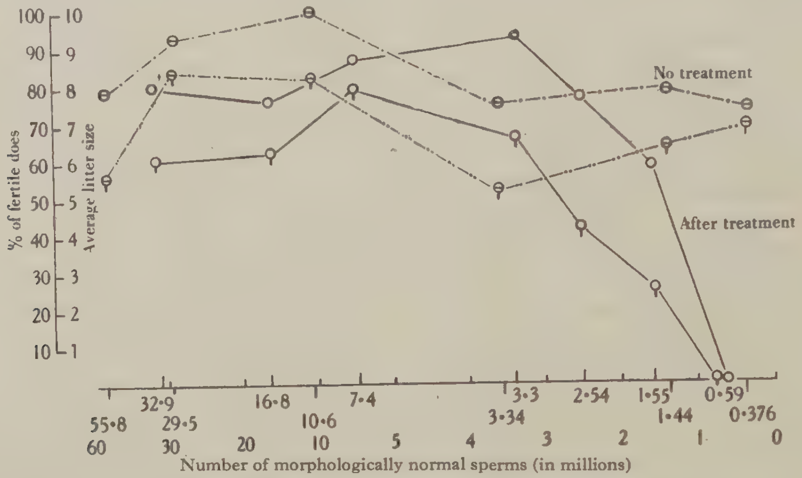

Fig. 2. Fertilizing capacity of spermatozoa after low temperature treatment of scrotal testes in comparison with that of spermatozoa without treatment of testes. ○, ⊖, percentage of fertile does. ♀, ⊖, average litter size.

Table 3. *Relative fertility of natural mating and artificial insemination*

	Data from Hammond (1934) (natural mating)	Inseminations (with normal samples or with treated samples containing more than 3×10^6 of normal sperms)	Inseminations (with treated samples containing less than 3×10^6 of normal sperms)
No. of matings or inseminations	327	127	53
% of fertile does	79·6	85·8	45·3
Litter size per mating or per insemination	5·3	6·6	2·2
Litter size per pregnancy	6·4	7·7	4·8

with natural mating. Table 3 presents fertility records of natural matings by Hammond (1934) and of artificial inseminations in the present paper. Of course, genetic and nutritional conditions may not be the same in the two groups, but from the evidence it would appear that artificial insemination is as efficient as natural mating. Furthermore, considering the fact that the number of sperms in a single ejaculate at normal mating is about 50–200 millions, and that the number required for full fertility with artificial insemination is about 1 million, it is quite clear that in normal mating a high proportion of spermatozoa are in excess of requirement. From 50 to 200 does might in fact be fertilized from a single ejaculation. These calculations confirm also those of Walton (1927) and Rowlands (1944) as to the approximate minimal number of spermatozoa required for fertilization.

DISCUSSION

It has already been pointed out in a previous paper (Chang, 1943) that after cold treatment of the testes, morphological disintegration of the spermatozoa takes a few hours to come to completion, but that physiological deterioration sets in much more quickly. The present experiments show how fertilizing capacity is affected and emphasize once more the error which may be involved if morphological normality is assumed to be an adequate criterion of fertilizing capacity.

The motility test of semen samples, based upon physiological activity, is sounder in principle, and has the merit of simplicity and convenience, but difficulties are involved in grading motility and determining the proportion of sperms in each grade. A physiological or biochemical measurement, like oxygen consumption or some other index of the metabolism of the spermatozoa, should be still more accurate as an estimate of the fertility of the male. The only absolute criterion of fertility is, however, the actual production of fertilized ova.

In the writer's experience a very small proportion of morphologically abnormal spermatozoa is present in the semen of healthy fertile domesticated animals. The order in which they appear with increasing number in different species is as follows: ram, male goat, bull, boar, stallion and rabbit. The spermatozoa of these animals are very uniform, particularly in the case of the ram. The high numbers of abnormals and the extreme abnormalities described in human semen have not been noticed in animal semen during 5 years' work at Cambridge. It may be that, in comparison with the animal, human spermatozoa and human spermatogenesis are more easily disturbed, or that the human environment is more liable to noxious fluctuations.

The abnormal sperms found in the semen of animals with impaired fertility due to disease or other disturbance may be different from those experimentally produced, and where only one factor may be involved in the animal with impaired fertility, there may be many causes of sterility operating simultaneously. Sterility may be due to disturbance of spermatogenesis, to chemical changes in the semen and to morphological and physiological changes in the spermatozoa. If, however, only a proportion of the spermatozoa is affected and a sufficient number of physiologically normal spermatozoa is present as well, the proportion of abnormal cells will not in any way reflect the actual fertility of the animal.

The fertilizing capacity of spermatozoa after cold treatment of the testes is definitely lower than that of sperms from untreated males. The impairment of fertilizing capacity due to morphological and physiological changes probably takes place in the epididymis rather than in the testis because both the intact sperms and the separated sperm heads are mature and well formed and because the proportion of abnormal sperms other than the separated sperm heads remains constant from the time of treatment until complete recovery, indicating that there has been no ill effect on spermatogenesis.

When ice was applied to the scrotum the actual temperature of the testis, as shown by thermocouples inserted in the tissues, was from +1 to +3° C. Although this temperature will cause mature sperms to disintegrate there was no harmful effect on spermatogenesis. When a freezing-mixture of ice and salt at a temperature of −11° C. was applied for 10 min., the temperature of the testis fell from −0·8 to −2·5° C., and this caused the seminiferous tubules to become atrophied. The interstitial tissue, however, and consequently the sex drive of the animal remained unimpaired. It is very interesting to note this differential reaction of different tissues to changes of temperature.

It has been demonstrated that the follicular granulosa surrounding the tubal eggs of the rabbit is dispersed in the presence of fairly large numbers of sperms by a heat-labile enzyme (Pincus & Enzmann, 1936) now identified as hyaluronidase (McClean & Rowlands, 1942). It is supposed that this dispersal must precede the penetration of the spermatozoon into the egg. The present investigation shows that when the number of sperms present is decreased, the average number of young born decreases at a more rapid rate than the percentage fertility, as shown in Fig. 1. It seems, therefore, that even if a certain critical concentration of hyaluronidase must be reached in order to liberate the ova from the mass of granulosa cells, the number of ova fertilized may still depend upon the chance meeting of sperm with ovum, and therefore dependent upon the number as well as upon the presence of some critical concentration of the enzyme.

The prodigality of sperm production is a striking phenomenon in the life of the male when compared with egg production in the female (Hartman, 1939). The prodigality of sperms in each ejaculate will be realized on comparing the number of sperms in a single ejaculate (50–200 millions) with the number required for full fertility (0·4 million). It seems that this large number in the ejaculate cannot be due entirely to the small chance of any one spermatozoon reaching the Fallopian tube. Exactly how many sperms are lost on their way up the Fallopian tube, and how many are required to disperse the granulosa cells, and how many are required to ensure penetration of the egg, are still questions which require answers.

SUMMARY

Doe rabbits were inseminated with sperm suspensions containing a known number of morphologically normal sperms, together with a known number of tailless sperm heads, produced artificially by cold treatment of the scrotal testes. The fertilizing capacity of the mixture, as determined by the percentage of fertile inseminations and the average number of young born, remained normal so long as a minimal number of normal sperms (viz. 3 millions) was maintained, irrespective of whether a high percentage of sperm heads (viz. 17–95) was present or not. The isolated sperm heads had obviously no fertilizing capacity. Partial fertility occurred when the number of morphologically normal sperms was less than 3 millions and sterility when the number was less than 1 million.

When a similar series of inseminations were done with spermatozoa from normal untreated males, the minimal number of normal sperms required for full fertility was 0·33 million, or rather less. This indicates that apparently normal sperms which are present in ejaculates after low temperature treatment of the testis are only morphologically normal and not physiologically normal. It is concluded from these results that the diagnosis of fertility or sterility, based only upon sperm morphology, may be inaccurate and misleading. A seminal specimen containing a high proportion of abnormal forms may be quite fertile provided it contains a sufficient absolute number of viable sperms, and conversely, spermatozoa which may appear morphologically normal may not be physiologically capable of effecting fertility.

The sex ratio of the offspring was not significantly disturbed when does were inseminated with sperms produced after cold treatment of the testes. The efficiency of artificial insemination as a method of ensuring high fertility has been demonstrated.

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DIFFERENTIATION OF ECTODERM IMPLANTED INTO THE PRIMORDIUM OF THE LIMB BUD IN THE AMPHIBIAN EMBRYO AND ITS INFLUENCE UPON THE LIMB*

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(With Plates 1 and 2)

It was shown by Brandt (1944) that phocomelias and atrophied limbs can be produced experimentally after deep implantation of embryonic ectoderm into the primordium of the limb bud in the tail-bud stage (Harrison's stage no. 35) of the amblystoma embryo. The following microscopical analysis of the operated specimens shows some new results which concern the histological changes of the implanted embryonic ectoderm within the tissues of the host embryo and their effect on the limb primordium.

MATERIAL AND METHODS

The material refers to sixty-three operated embryos. Out of this number eight were cut in serial sections of 10μ thickness and stained with haematoxylin and eosin. The reconstructions, the photographs of which are shown on Pl. 1, were made by the following method: The serial sections were projected with the Leitz panphot projecting apparatus at a 100 times magnification on white paper, and the outlines of the structures of each section and some superficial landmarks traced. The tracings were mounted on wax plates of 1 mm. thickness and cut out with a sharp knife. The cuttings were placed on top of each other and their surfaces smoothed and stuck together.

Description of the microscopical structures

(1) *Case* 959. A small piece of embryonic ectoderm from the tail of a donor embryo was implanted deep into the primordium of the limb of an amblystoma embryo (stage 35, Harrison). Four weeks later a stump had developed at the site of operation. The distal end of the stump was curved ventrally and ended in a pointed tip, but no fingers were visible. The relation of the total length of the right stump to the length of the body of the larva was 0·7 : 16·2; the corresponding relation of the normal left limb was 1·4 : 16·2. Four weeks later the operated atrophied right limb had not developed further.

Microscopical structures of the operated limb. There was no abnormal structure visible in the proximal part of the limb. The head of the humerus and the socket of the glenoid cavity of the scapula were well developed. The distal end of the cartilage of the humerus was attached at an angle of about 90° to the cartilages of the forearm without forming a joint. The cartilaginous elements of the forearm and those of the carpus and fingers were irregularly scattered throughout the whole length of the forearm. The structure inside the distal pointed end of the limb was formed by a single cartilaginous element.

Microscopical structures of the implant. The implanted ectoderm was attached as a triangular protuberance along the ventral surface of the distal half of the upper arm and along the whole length of the forearm. The implanted ectoderm covered the pointed distal end of the limb. No epithelial remains were visible inside the tissues of the limb.

(2) *Case* 963. Same operation as case 959. Twenty-six days after the operation a phocomelias had developed (Brandt, 1944, pl. 4, fig. 1), and at its distal end four fingerlike elevations were visible.

Microscopical structures of the operated limb. The reconstruction from the serial sections (Pl. 1, reconstruction 1 A, B) showed the humerus lying on the body wall directed backwards. The humerus, by comparison with its fellow, was of normal length. The head of the humerus surrounded by a capsule lay in a well-formed cavity on the scapula. The normal shoulder-joint showed no difference from that of the operated side. At the medial side of the proximal part of the humerus lay a small piece of cartilage measuring 40μ in length (Pl. 1, fig. 2). The elbow-joint was just in the area where the limb was attached to the body wall. The elbow formed an angle of 90° with the humerus. The cartilages of the forearm and of the carpus were dislocated and irregularly scattered throughout the core of the forearm (Pl. 1, figs. 2, 3). A single rod of cartilage, probably representing a phalanx, formed the hook-like free distal end of the limb. The muscles of the wall of the thorax on the operated side were dislocated, and there was a mass of muscles on the lateral side of the mesonephros separated from the main part of the muscles. The dislocated muscular

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tissue lay in the area of the suprascapula, which was absent. The tubules of the mesonephros in that area were reduced in number (Pl. 1, fig. 1).

Microscopical structures of the implanted ectoderm. The implanted ectoderm was attached to the body wall, covering the area where normally the limb developed and it had formed three fingerlike elevations. The elevations consisted of a mesodermal core covered with ectoderm. No epithelial cords or cysts were visible inside the elevations.

(3) *Case* 975. Same operation as in cases 1 and 2. Four weeks after the operation the left limb was well developed, possessing upper arm, lower arm, hand and fingers. The operated right limb was a pointed stump (Brandt, 1944, pl. 4, fig. 2). The stump did not develop further and it showed later an oedematous swelling at its root.

Microscopical structures of the operated limb. The scapula, the humerus and the shoulder-joint of the operated limb were normal in shape, size and structure. The distal parts of the limb, both the skeletal elements of the forearm and the cartilages of the carpus, were dislocated. The fingers were represented by two rudimentary phalanges embedded in the tissue at the pointed distal end of the stump.

There was a dislocation and a disturbance of development of some parts of the mesonephros, the muscles and the gut near to the area of operation. At the ventral side of an enlarged tubule of the mesonephros (Pl. 1, fig. 4) lay an isolated piece of cartilage which did not possess any relation to the cartilage of the shoulder-girdle. This piece of cartilage could be found in 29 sections of 10 μ. Along the ventrolateral side of the cartilage there was a blood vessel and some scattered bundles of muscle tissue. In sections further caudally at the level of the liver two small tubules appeared (Pl. 1, fig. 5) within a fold of mesenchyme projecting into the abdominal cavity. The distal ends of both the tubules were connected caudally with the gut.

No remains of the implanted ectoderm could be found in the operated area.

(4) *Case* 989. Same operation as in cases 1–3. Three weeks after the operation the limb was atrophied showing a ventrally bent stump. The stump did not grow further during the next 5 weeks.

Microscopical structures of the operated limb. The humerus inside the tissues of the wall of the body was reduplicated. Two heads were present (Pl. 1, fig. 6). The uppermost part of the head of the humerus developed in an area of proliferation of cells of the outer third of the scapula. More caudally the proliferation formed a bulge which finally became separated from the mass of the scapula. A small cavity of a joint appeared between the scapula and the adjacent part of the head of the humerus, but there was no socket visible in the scapula. Apart from this head of the humerus (no. 1) there appeared another smaller head of humerus (no. 2) some distance away from both the scapula and humerus (no. 1). The head of humerus 2 lay free in the embryonic mesenchyme (Pl. 1, fig. 6). The shafts of both humeri differed from each other. The distal end of humerus 1 ended free in the tissue of the lateral body wall without fusing with the free limb. Humerus 2, which is not related to the scapula, was very thin in its middle third. This part of humerus 2 lay just in the area where the humerus entered the free limb outside the body wall (Pl. 2, fig. 7). Distal to this thin part the humerus thickens again, showing well-differentiated cartilaginous cells. To the shafts of both humeri some muscle fibres are attached. The distal thickened part of humerus 2 presents a pointed free end which was surrounded by a densely packed cluster of cells (Pl. 2, fig. 8), and adjacent to this another rod of cartilage appears which runs parallel to the axis of the shaft of the humerus (Pl. 2, fig. 8). This rod represented the only skeletal element of the forearm for the carpal elements and the phalanges were absent.

Microscopical structures of the implanted ectoderm. The only remains of the implanted ectoderm were attached to the distal end of the pointed limb, the core of which consisted of mesenchyme.

(5) *Case* 990. The embryo was operated on at stage 37 (Harrison) in the same way as cases 1–4. Four weeks after the operation the operated limb was shorter than the normal limb, and its longitudinal axis was directed dorsoventrally. The distal end of the stump was thickened and bifurcated. Two other fingerlike processes were inserted along the ulnar border of the distal part of the limb.

Microscopical structures of the operated limb. The scapula, the humerus and the shoulder-joint were normal. The thickness of the distal end of the limb was caused by an irregular arrangement of the carpal elements of the hand (Pl. 2, fig. 9). The bifurcation at the distal end consisted of the implanted ectoderm and an isolated phalanx projecting outside as a fingerlike elevation (Pl. 2, fig. 9). The processes along the ulnar border of the distal end of the limb consisted of three phalanges, one of them so short that it could not be seen by the naked eye.

Microscopical structures of the implanted ectoderm. The implanted ectoderm formed a fingerlike elevation at the distal end of the limb, and it possessed a mesenchymal core (Pl. 2, fig. 9). No other remnant of the implant was visible.

(6) *Case* 1000. The embryo was operated on at stage 37 (Harrison) after method 3 (implantation along a vertical line, Brandt, 1944). Five weeks after the operation the implanted ectoderm was attached along the whole length of the radial border of the limb. The proximal part of the limb was reduced in length when compared with the normal limb, and at the distal end were three fingerlike processes visible (Brandt, 1944, pl. 4, fig. 5).

Microscopical structures of the operated limb. The scapula was dislocated deep into the muscles which lay along the wall of the abdominal cavity (Pl. 2, fig. 10). The scapula and the suprascapula were represented by some isolated cartilaginous pieces, the gaps between them being filled with bundles of muscle fibres originating from the muscles of the abdominal wall (Pl. 2, fig. 10). There was no shoulder-joint. The head of the humerus had not developed from a centre of proliferation at the side of the scapula but as a prolongation of cartilaginous tissue at the dorsal edge of the most ventrally situated isolated piece of the scattered scapula. The distal half of the humerus was thicker than the proximal half. Both halves were thinner than the corresponding parts of the normal humerus on the other side. The cartilaginous elements of the forearm and of the carpus were scattered throughout the distal half of the limb. Each of the three very short fingers contained its cartilaginous phalanges. The tips of the fingers were free.

Microscopical structures of the implanted ectoderm. No epithelial cords or cysts were visible inside the limb. The implant was attached as a notched protuberance along the whole length of the radial border of the limb. The protuberance possessed a mesenchymal core. The uppermost part of implanted ectoderm was attached at the area of the scattered pieces of scapula (Pl. 2, fig. 10).

(7) *Case* 1001. The embryo was operated after method 3 (Brandt, 1944, implantation of ectoderm along a vertical line) at stage 37 (Harrison). Five weeks after the operation the implanted ectoderm was attached along the whole length of the radial border of the limb, ending in a free fingerlike process (Brandt, 1944, pl. 4, fig. 6). The operated limb was half as long as the normal and ended in a long pointed tip.

Microscopical structures of the operated limb. In the area of the scapula where normally the shoulder-joint develops, the heads of two humeri were visible. A normal socket was not present. The mass of cartilaginous cells which formed the head of one of the humeri was attached at the outer surface of the scapula (humerus 1), the other was not related to the scapula (humerus 2), but lay in close relation to the head of the other humerus. The proximal end of humerus 2 was normal in size and shape, but its diameter soon diminished (Pl. 2, fig. 11). The nuclei of the cartilaginous cells of humerus 2 became translucent and were not so deeply stained as those in humerus 1. The distal end of humerus 2 appeared finally as a small thin rod attached along the outer surface of the stronger humerus 1. The shaft of humerus 2 ended free within the tissues of the body wall; the shaft of humerus 1 was equal in diameter to the humerus of the normal left limb and entered into the free part of the limb outside the body. There was no elbow-joint present, and the skeletal element of the forearm was attached at an angle of 90° to the distal end of the humerus (Pl. 1, reconstruction 2). One skeletal element only was present in the forearm (Pl. 2, fig. 12), the place of the second was taken by the implanted ectoderm (Pl. 2, fig. 13). The muscles of the forearm ran parallel to the long axis of this skeletal element. At the distal end of the forearm three cartilaginous carpal elements were visible. Distal to the carpus there was a thin, very long rod of cartilage representing a phalanx of abnormal length attached at an angle of 90° at the carpus (Pl. 1, reconstruction 2). This cartilaginous rod was surrounded by masses of muscles showing an enormous number of densely packed nuclei. The muscles followed the course of the cartilaginous rod to which they are attached all around.

Microscopical structures of the implanted ectoderm. The implanted ectoderm was attached along the whole length of the radial (pre-axial) border of the proximal part of the limb (Brandt, 1944, pl. 4, fig. 6). The ectoderm had many layers and surrounded a mesenchymal core. A part of the ectoderm was invaginated into the core, separating some bundles of muscles from each other. The distal end of the invaginated epithelial cord became hollowed out and at several points surrounded cysts of different sizes filled with fluid (Pl. 2, fig. 13). In one section there were four cysts visible (Pl. 2, fig. 13) which lay close to each other; their cavities did not anastomose. At the areas which were taken by the epithelial cords and cysts no skeletal elements of the limb had developed (compare Pl. 2, figs. 12, 13). The mesenchymal core of the implanted ectoderm was vascularized; the vessels ran parallel to the long axis of the cords.

(8) *Case* 1005. The same operation was performed at the same stage of development as in the seventh case. Nineteen days after the operation the implanted ectoderm was attached to the proximal part of the limb. There were no movements visible in the shoulder-joint. Five weeks after the operation the normal unoperated right limb showed an elbow and three fingers on the hand, but the operated limb did not show an elbow and the limb was stiff and stunted at its end. The operated limb had a normal number of fingers which, however, were irregular in position. The first finger was directed obliquely backwards and downwards, the second and third fingers were fused at their bases (syndactylias), the fourth finger was situated below the third, and the fifth finger was very small. Measurements of the lengths of the parts of the limbs taken 2 months after the operation showed:

	mm.
Length of the normal right upper arm	2·0
Length of the normal right forearm (elbow to tip of 3rd finger)	3·3
Length of the operated left upper arm	1·0
Length of the operated left forearm	3·1

The measurements indicate a shortening of the length of the upper arm of the operated limb at half the length of that of the normal upper arm.

Microscopical structures of the operated limb. At the cranial end of the scapula near to the insertion of the head of the humerus an accessory piece of cartilage could be seen which pierced the muscles of the abdominal wall, entered the abdominal cavity and caused a slight impression on the surface of the liver (Pl. 2, fig. 14). More caudal to that area the head of the humerus lay in a well-formed glenoid cavity and a capsule was attached to both the humerus and scapula. The cartilaginous cells of the distal part of the humerus were pale and notched, and their nuclei were not as deeply stained as those of the normal humerus. The perichondrium appeared as a thickened reddish ring around the cartilaginous rod of the humerus. The cartilages of the forearm and carpus did not show any alteration. The phalanges were dislocated and epithelial cords and a cyst lay in close relation to them (Pl. 2, fig. 15). Cross-sections through some phalanages and a longitudinal section through the abnormal long first phalanx lay close to each other in the same section (Pl. 2, fig. 15). There was no phalanx present on the fourth finger.

DISCUSSION

The differentiation of ectoderm of the amphibian embryo (stages 35–37, Harrison) after its implantation into the primordium of the limb of a host embryo of same stage of development was threefold:

(1) The ectoderm lay deep in the mesenchyme of the host and developed either solid epithelial cords or cysts (cases 1001 and 1005).

(2) The ectoderm was attached at the outer surface of the limb and formed irregular ectodermal elevations which included a mesenchymal core (case 963).

(3) The ectoderm could act upon some structures of the proximal part of the primordium. In these cases there was no remnant of ectoderm visible in the tissues (cases 975 and 989).

The influences of the implanted ectoderm on the primordium were as follows:

(1) Solid epithelial cords and cysts prevented the development of those structures of the limb which normally develop at the areas taken by the implant. The result was atrophy of special parts of the limb, a local deficiency (cases 989 and 1001).

(2) When the implanted ectoderm was attached outside at the body wall in the area where normally the limb developed, the ectoderm formed irregular elevations which possessed a mesenchymal core. The elevations prevented a normal outgrowth of the limb, and a phocomelias could be produced experimentally. The whole length of the humerus developed inside the tissues of the body (case 963). The cartilages of the forearm and of the carpus were irregularly scattered throughout the free part of the limb, which was covered outside by the ectoderm of the donor embryo. Either the development of the fingers was suppressed and only one finger could be present forming a hooklike appendix at the distal free end of the limb, or rudiments of some phalanges were enclosed in the tissue of the distal end of the atrophied limb (case 975). In case 1000 the ectoderm was attached along the whole radial border of the limb as a long elevation with a notched surface. The cartilages of the forearm and carpus were irregularly scattered throughout the tissues of the forearm, and three very short rudiments of fingers were present which formed the distal end of the atrophied limb. In case 1000 three very short rudiments of fingers appeared; in case 1005 one finger was much longer than normal and the others were short and dislocated; two of them showed syndactylias. When the ectoderm was attached at the distal tip of the hand it almost completely suppressed the development of the fingers. The carpal elements were dislocated.

(3) The reactions of the proximal part of the limb were as follows:

(*a*) *Scapula.* Some bundles of muscle tissue were dislocated into the area of the suprascapula. The dislocated muscle prevented the development of the suprascapula (case 963). The scapula was divided into three isolated pieces, and a very small head of the humerus was attached at one end of the middle piece without showing the centre of proliferation of cartilage which normally appeared during the development of the humerus (case 1000).

An isolated supernumerary piece of cartilage protruded into the abdomen (case 1005). This deformity was similar to the breaking up of the scapula itself into three isolated pieces as in case 1000.

(*b*) *Humerus.* The proximal half of the humerus was thinner in diameter than the distal half (case 1000).

The humerus was shorter than normal and showed, in its distal half, notched, cartilaginous cells with pale nuclei.

In two cases, 1001 and 989, a reduplication of the humerus had developed. There was no remnant of implanted ectoderm visible on the reduplication. The ectoderm possibly pressed upon the primordium of the humerus causing a twinning. In 1925, Brandt showed that during the earliest phase of determination (the 'reversible phase') the primordium of a limb can produce a reduplication. The present cases showed that this phenomenon could be restricted to one unit of the limb (the humerus). We can therefore say that in stages 35–37 the primordium of the humerus possesses the reversible phase of determination and can produce a reduplication after the application of the methods described.

(*c*) *Forearm, carpus and fingers.* In the forearm one skeletal element only was present (radius or ulna), and in the region where the skeletal element normally developed epithelial cords or cysts were present (case 1001). The carpal elements and the skeletal elements of the hand can be irregularly scattered throughout the tissues when the implanted ectoderm was attached at the distal part of the limb. The fingers were reduced in number and one finger might be abnormally long.

SUMMARY

1. A microscopical analysis was made concerning the differentiation of ectoderm cut from the tip of the tail-bud of an amphibian embryo (*Amblystoma mexicanum*, stages 35–37, Harrison) after its implantation into the primordium of the limb-bud of a host embryo 3–5 weeks after operation.

2. The ectoderm which lay deep in the tissues of the limb differentiated either into solid epithelial cords or into cysts.

3. The ectoderm which was attached outside the limb differentiated into notched ectodermal elevations which included a mesenchymal core.

4. A microscopical analysis was made concerning the development of deformities of limbs as the result of the operation.

5. The scapula may be divided into isolated pieces, bundles of muscle fibres separating the pieces from each other.

6. A supernumerary piece of cartilage can develop close to the cartilage of the scapula.

7. The suprascapula may be absent and its place taken by a mass of muscle fibres.

8. A phocomelias may be produced when the whole length of the humerus and the elbow-joint lies inside the body wall. In this case the implanted ectoderm covers the area where the limb would normally develop.

9. The humerus may be reduplicated.

10. The humerus may be too short.

11. The proximal half of the humerus may possess a diameter different from that of the distal half.

12. One skeletal element only of the forearm (radius or ulna) may be present when the place which would normally be occupied by one of these elements was taken by implanted ectoderm.

13. The elements of the carpus and of the hand may appear irregularly scattered throughout the tissues of the distal part of the limb. In these cases the implanted ectoderm was attached to the surface of the distal end of the limb.

14. The fingers can show: (*a*) abnormal positions, (*b*) abnormal numbers, (*c*) syndactylias, (*d*) one finger too long, others too short.

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EXPLANATION OF PLATES

Plate 1

Reconstruction 1 (case 963). A. Reconstruction of implanted ectoderm attached at the outer surface of the area where the limb develops. B. Reconstruction of structures within the body wall. *a*, fingerlike elevations, formed by implanted ectoderm; *b*, scapula; *c*, humerus inside the body wall; *d*, inner aspect of body wall; *e*, attachment of distal end of the humerus to the forearm within the body wall; *f*, skeletal element of forearm embedded in the mesenchymal core of the implanted ectoderm; *g*, cartilage of a phalanx which forms the hook-like free distal end of the limb.

Reconstruction 2 (case 1001). *a*, epithelial cords; *b*, cysts; *c*, free fingerlike process of implanted ectoderm; *d*, the only cartilaginous element (radius or ulna) of the forearm; *e*, carpal elements and rudiments of phalanges; *f*, abnormal long phalanx; *g*, humerus 1.

Fig. 1 (case 963). Slide 10, row 2, section 3. Cross-section whole larva. 22 ×. *a*, mesonephric tubules reduced in number; *b*, dislocated muscle, preventing development of suprascapula; *c*, suprascapula at the normal side; *d*, implanted ectoderm.

Fig. 2 (case 963). Slide 11, row 1, section 9. Cross-section operated limb. 70 ×. *a*, humerus; *b*, accessory piece of cartilage (both *a* and *b* lie inside the body wall); *c*, carpal elements; *d*, implanted ectoderm.

Fig. 3 (case 963). Slide 11, row 3, section 3. Cross-section operated limb. 70 ×. *a*, humerus; *b*, radius or ulna; *c*, carpal elements; *d*, proliferating masses of muscles; *e*, implanted ectoderm.

Fig. 4 (case 975). Slide 12, row 3, section 6. Cross-section operated side of the larva, 4 weeks after operation. 35 ×. *a*, enlarged mesonephric tubule; *b*, isolated piece of cartilage; *c*, dislocated muscular tissue; *d*, shoulder-girdle; *e*, liver.

Fig. 5 (case 975). Slide 13, row 1, section 6. Cross-section operated side of the larva, 4 weeks after operation. 35 ×. The section is caudal to section of fig. 4. *a*, two tubules in a fold, projecting into the abdominal cavity; *b*, humerus; *c*, scapula; *d*, liver.

Fig. 6 (case 989). Slide 10, row 2, section 4. Cross-section proximal part of the limb. 35 ×. *a*, reduplicated humerus 1; *b*, reduplicated humerus 2; *c*, muscle tissue.

Plate 2

Fig. 7 (case 989). Slide 10, row 4, section 3. Cross-section whole larva. 18·5 ×. *a*, distal end of reduplicated humerus 2, entering free limb. Its diameter is smaller than that of the normal humerus on the other side (*b*). *c*, implanted ectoderm.

Fig. 8 (case 989). Slide 11, row 2, section 5. 70 ×. *a*, cluster of cells beyond the distal free end of humerus 2; *b*, the only skeletal element of the forearm; *c*, epithelial cord; *d*, implanted ectoderm.

Fig. 9 (case 990). Slide 12, row 3, section 7. Cross-section distal end of the limb. 70 ×. *a*, implanted ectoderm; *b*, isolated phalanx; *c*, radius; *d*, ulna; *e*, carpal elements; *f*, muscle fibres.

Fig. 10 (case 1000). Slide 10, row 4, section 12. Cross-section proximal part of the limb. 15 ×. *a*, shoulder-girdle, split into three isolated pieces of cartilage; *b*, muscle tissue intercalated between the split parts; *c*, abdominal cavity close to the dislocated shoulder-girdle; *d*, implanted ectoderm.

Fig. 11 (case 1001). Slide 11, row 4, section 2. Cross-section through the proximal part of the limb. 100 ×. Reduplicated humerus; both humeri are united by a bridge of cartilage. *a*, humerus 1; *b*, humerus 2 (thinner in its diameter).

Fig. 12 (case 1001). Slide 13, row 3, section 10. Longitudinal section through forearm, 30 sections below the section on fig. 13. 70 ×. The forearm is attached at an angle of 90° to the humerus. *a*, cyst in continuation to the cyst *a* in fig. 13; *b*, the only skeletal element of the forearm; *c*, three carpal elements; *d*, humerus.

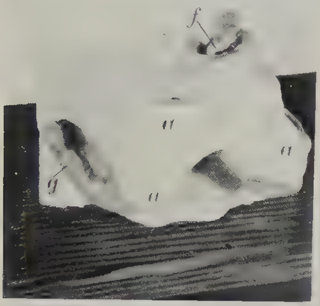
Fig. 13 (case 1001). Slide 13, row 1, section 10. Longitudinal section through the forearm, 30 sections above the section on fig. 12. 70 ×. *a*, cyst, in continuation to cyst *a* on fig. 12; *b*, epithelial cord replacing skeletal element; *c*, cysts; *d*, humerus.

Fig. 14 (case 1005). Slide 20, row 1, section 6. Cross-section through the scapula of operated side. 100 ×. *a*, scapula; *b*, area where the head of the humerus develops; *c*, coracoid; *d*, supernumerary piece of cartilage; *e*, liver.

Fig. 15 (case 1005). Slide 27, row 2, section 5. 70 ×. Longitudinal section through the distal end of the operated limb. *a*, dislocated phalanges, the axes of which form an angle of 90° with phalanx *b*; *b*, phalanges of an abnormally long finger; *c*, epithelial cord; *d*, cyst.

The photographs were made by the technician of the department, Mr W. J. Pardoe.

Reconstruction 1A



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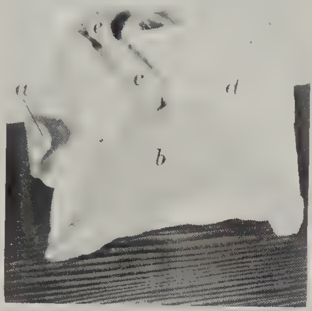

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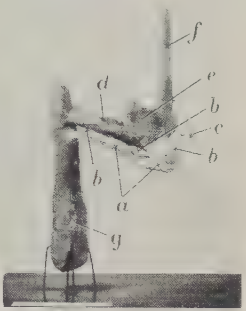

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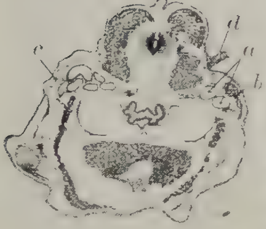

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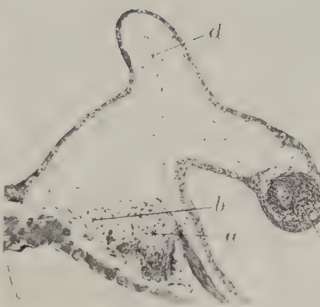

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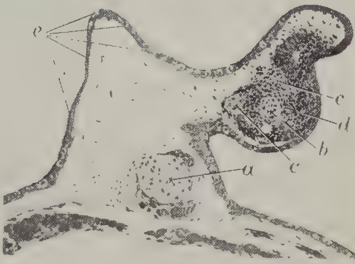

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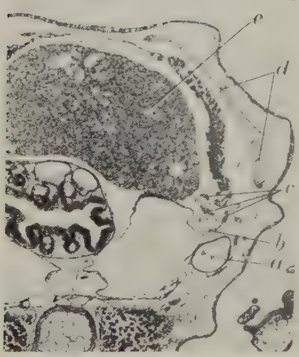

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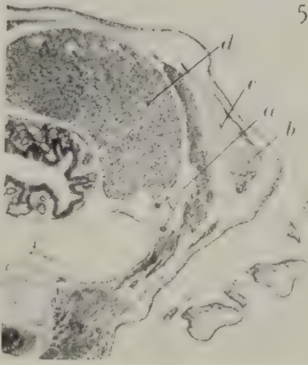

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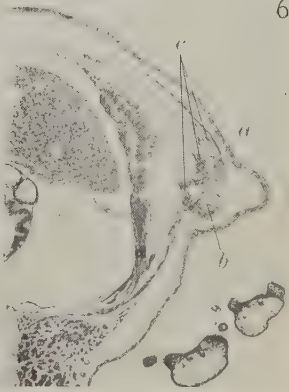
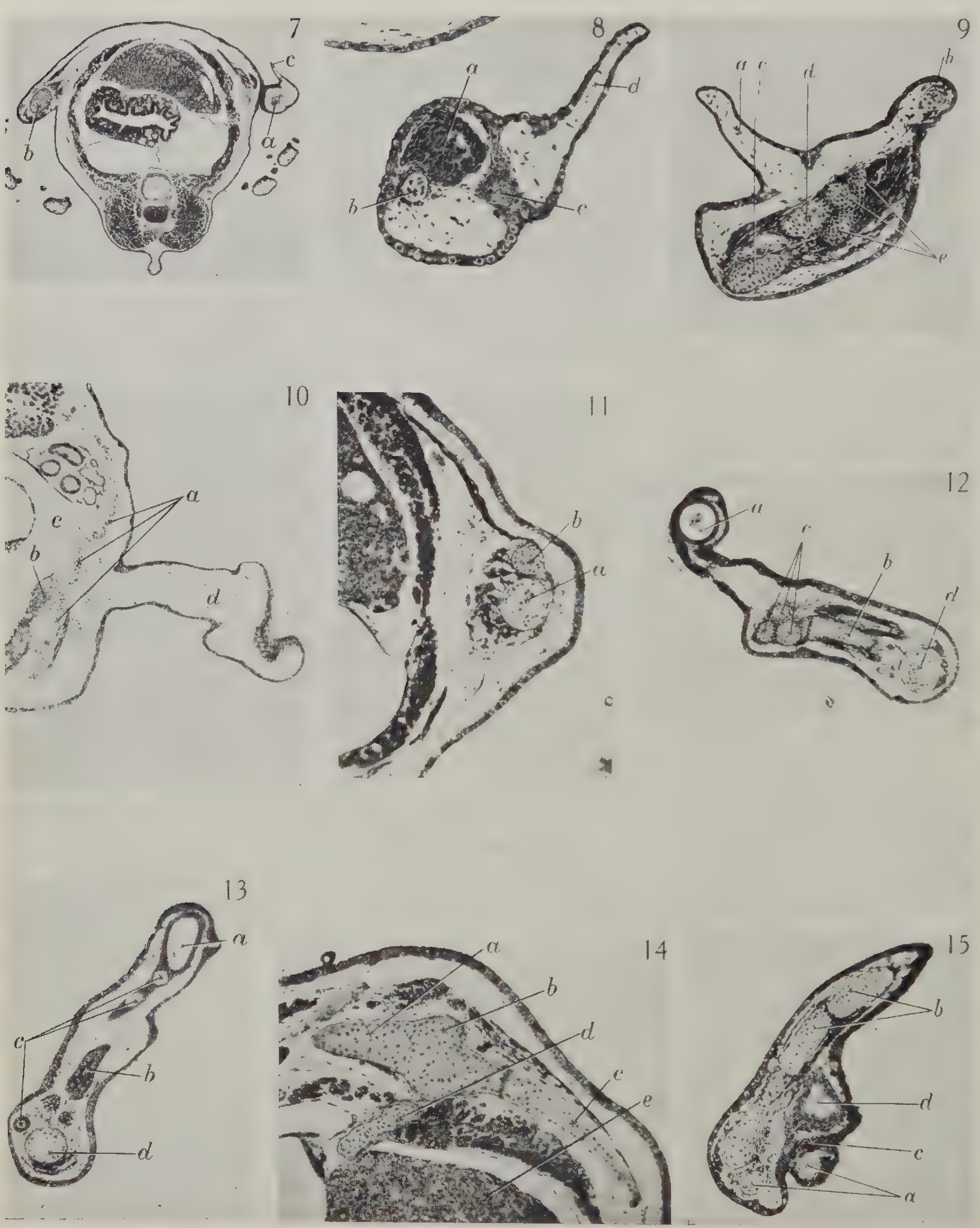


BRANDT—ECTODERM IMPLANTED INTO THE LIMB PRIMORDIUM



BRANDT—ECTODERM IMPLANTED INTO THE LIMB PRIMORDIUM

PERIPHERAL NERVE AND REGENERATION IN CRUSTACEA. II

By A. E. NEEDHAM, *Department of Physiology, University of Manchester*

(*Received* 1 *October* 1945)

In the previous paper of this title (Needham, 1945) denervation of a thoracic limb of the isopod *Asellus aquaticus* at the time of amputation was shown to result in a marked retardation of regeneration, as compared with the partner limb amputated, without denervation, at the same time. In the present work it was hoped to throw more light on this neurotropic effect by experiments involving denervation at different times relative to amputation. Schotté & Butler (1944) find that the peripheral nerve is necessary for the early stages but not for the later stages of regeneration of amphibian limbs. In one series of the present experiments denervation was performed 5 days after amputation, and in a second series after normal regeneration for one instar, that is, at the first appearance of an externally visible regenerate, an average of 10 days after amputation. Such a regenerate is already partly functional, so that the possibility must be envisaged that denervation might lead to some disuse-atrophy in addition to its possible effect on regenerative growth; in a third series, therefore, the normally growing limb was denervated (without amputation), in order to obtain some measure of the extent of this disuse-atrophy. The results of this third series may include an effect of denervation on normal (i.e. non-regenerative) growth also. In a fourth series the limb was denervated 5 days before amputation, the object being to ascertain whether tissue denervated some time previously to amputation and undergoing the consequent atrophic changes is capable of developing a regeneration blastema at the surface of amputation, and of regenerating at the normal rate as soon as the regenerating nerve reaches that surface (cf. Needham, 1945, p. 146).

Table 1. Asellus aquaticus. *Inhibitory effect of denervation, at various stages relative to amputation, on regeneration of a thoracic limb; the partner limb, as control, regenerating under normal conditions, for one instar*

Series no.	Nature of the experiment
1	Control. Wound, not involving peripheral nerve, applied to limb base.
2	Denervation simultaneous with amputation
3	Denervation 5 days *after* amputation.
4	Denervation 10 days (one instar) after amputation.
5	Denervation of normally growing limb, without amputation.
6	Denervation 5 days *before* amputation.

Series no.	No. of individuals	Mean abdomen width	Mean control limb length	Mean experimental limb length	Mean difference between control and experimental limb length	Probable error of mean difference	Figures appropriate to common mean abdomen width of 100 units		
							Control limb length	Experimental limb length	Difference between control and experimental limb length
1	18	136	155·7	145·7	9·95	3·93	114·5	107·1	7·31
2	18	140	170·0	112·7	57·26	6·36	123·0	80·5	40·90
3	18	124	157·8	112·6	45·15	3·66	127·1	90·7	36·41
4	23	96	194·6	170·8	23·81	2·33	201·9	177·2	24·70
5	25	116	265·9	237·9	28·33	3·29	229·0	204·9	24·40
6	18	119	132·6	113·0	20·21	3·25	111·4	95·0	16·98

All dimensions in mm. × 56.

The four series will be referred to as series 3, 4, 5 and 6 and the two of the previous paper as series 1 and 2 (Table 1).

METHOD

All essentials of procedure were as in the previous paper, and the same tests were applied for the significance of the mean difference in length between experimental limb and control limb of the other side of the body. Data for about twenty individuals were obtained in each series. The limbs concerned were measured after the first ecdysis following the final operation, that is, following denervation in series 3, 4 and 5, and amputation in series 6.

RESULTS

The relevant data, together with those for series 1 and 2, are summarized in Table 1. The mean body size (measured by abdomen width) varied somewhat in the different series, and it is useful, for purposes of comparison, to convert all measurements to those appropriate to a common abdomen width of 100 units, assuming an approximately linear relation between abdomen width and limb length, at corresponding stages of regeneration or of normal growth.

Denervation is seen to have a clearly and uniformly significant retarding effect on regeneration at all stages. The magnitude of the effect, however, decreases progressively with increasing interval between amputation and denervation (series 2, 3 and 4), in spite of the fact that complete reinnervation of the growing limb must occupy an increasingly long time. The decrease is probably logarithmic rather than linear, so that the effect on a one-instar regenerate (series 4) is already no greater than on a normally growing limb (series 5). The decrease probably does no more than parallel the decrease in the rate of normal regeneration itself.

It is not possible to estimate the effect of denervation on normal, non-regenerative growth, though some such effect seems *a priori* probable, as in the Amphibia (Hamburger, 1928). Much of the effect of denervation in both series 4 and 5 was undoubtedly due to disuse-atrophy. There was frequently an evident shrinkage of the denervated limb so that it became significantly shorter than the control limb, even before the next ecdysis, that is, before there could be any growth of the control limb. The available data for pre-ecdysal limb lengths indicate a mean figure of about 14 units for the control-experimental difference in limb length, in both series, that is, between one-half and two-thirds of the total, post-ecdysal difference. The increase in the difference at ecdysis is also approximately the same in both series, and certainly not greater in 4 than in 5. By contrast, the increase in limb length at ecdysis was at least twice as great in 4 as in 5 (57 : 27 units for the control and 47 : 16 units for the experimental limb); if, as suggested above, retardation of growth is roughly proportional to the growth rate itself, then it must form a small fraction of the total observed control-experimental difference in series 4 and 5.

In series 6 there was a clearly significant retardation of regeneration, but of much smaller magnitude than in series 2–5. Tissue denervated 5 days previously to amputation appears to be capable of developing a regeneration blastema, which probably commences normal regeneration as soon as the nerve, already in process of regeneration, reaches the surface of amputation. The data are in accordance with this assumption. The effect in this series was greater and more significant than in series 1: that is to say, destruction of other tissue, at the time of amputation, affects regeneration less than does the absence of nerve for the short period of 2 or 3 days.

The visible results of denervation of a limb are of incidental interest. The limb is not usually cast off: autotomy is a reflex act and is abolished by denervation. Otherwise series 4, 5 and 6 would be impracticable. The limb is paralysed, usually with the proximal segments in extension. The muscle proteins become visibly opaque, and there is a varying degree of atrophy accompanied by shrinkage and by crumpling of the exoskeleton. Rarely, the most distal segments degenerate completely and subsequently regenerate; when this occurred in series 4 and 5 the figures for these segments, for both control and experimental limb, were omitted from the estimate of total limb length. Denervated limbs are less easily withdrawn from the exuvia at ecdysis than are normal limbs. It is probable that the disappearance of the opacity and shrinkage of the tissues is synchronous with reinnervation and the recovery of motor function.

It seems probable that in early stages of regeneration, also, there is no marked degeneration following denervation, and that regeneration is resumed in the regeneration bud as soon as it is reinnervated. Otherwise the control-experimental difference in series 3 could scarcely be less than in series 2 (for in series 3 the control limb regenerated for 5 days before denervation), or the experimental limb length relatively so much greater (90·7 : 80·5 units).

DISCUSSION

The main conclusions to be drawn from the experiments are that the extent of inhibition of regeneration by denervation depends on (1) the time required for the regenerating nerve to reach the regenerating tissue, and (2) the normal rate of regeneration at the time of denervation. Probably little degeneration follows denervation, and regeneration is resumed where it was interrupted.

It cannot be decided from the present data if denervation of a limb in the later stages of regeneration or during normal growth has any retarding effect on growth as distinct from the effect of disuse-atrophy. Evidence for this might be sought in one of the larger species of Crustacea, by section of other components of the peripheral nerve leaving the motor component intact, if, as in Amphibia, it is the sensory nerve (Singer, 1943), or the autonomic supply to the limb (Schotté, 1926) which is responsible for the tropic effect on growth. Whatever the nature of the effect of denervation in these stages, however, there is no doubt that the peripheral nerve is essential for the normal condition and behaviour of the limb, and there is probably no stage during which the peripheral nerve is not of vital importance.

The close similarity between the mean length of the experimental limb in series 2, 3 and 6 is striking but probably only a fortuitous consequence of the differences in mean body size in the three series. The figures appropriate to a common mean abdomen width differ considerably (Table 1). On the other hand, the mean length of the control limb for the four series with first instar regenerates (1, 2, 3 and 6) shows much less variation when this difference in mean abdomen width is allowed for. Normal regeneration is thus fairly constant in amount in an instar (cf. Przibram, 1909, p. 114). The mean control limb length for series 2 and 3 is, nevertheless, somewhat greater than for series 1 and 6, in which the experimental limb was less inhibited, thus supporting the unconfirmed suggestion of the previous paper (1945, p. 145) that the regeneration of the control limb is accelerated in proportion to the degree of inhibition of the experimental limb. Statistical tests of this have not been applied to the present data.

The results of series 6 suggest that in Crustacea the co-operation between a transient local 'wound factor' and an 'emanent' factor, probably due to the nerve supply, is not so important for regeneration as in Amphibia (Needham, 1941, p. 81). In this series there could scarcely be any normal nerve tissue at the surface of amputation within 2 days of the time of amputation. Reinnervation would seem to initiate regeneration whatever the conditions of amputation and denervation. Experiments have, in general, failed to reveal any significant stimulus to regeneration by a wound factor in this crustacean.

SUMMARY

1. The peripheral nerve is probably essential for all stages of regeneration of thoracic limbs in the crustacean *Asellus aquaticus*. The inhibitory effect of denervation decreases, however, with the rate of regeneration itself.

2. When a limb reaches the functional stage denervation leads to some degree of disuse-atrophy, which masks the possible neurotropic effect on late stages of regeneration and on normal growth.

3. A limb denervated previously to amputation is capable of forming a regeneration blastema at the surface of amputation, and normal regeneration probably begins as soon as the regenerating nerve reaches that surface.

4. There is probably no general degeneration following denervation at any stage of regeneration or of normal growth, and growth is probably always resumed with reinnervation.

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A CRITICAL STUDY OF TECHNIQUES FOR DETERMINING THE CYTOLOGICAL POSITION OF ALKALINE PHOSPHATASE

By J. F. DANIELLI, *from the Departments of Biochemistry and Zoology, Cambridge*

(*Received* 17 *September* 1945)

(With Plate 3)

INTRODUCTION

Alkaline phosphomonoesterase is the enzyme which catalyses the hydrolysis of simple esters of orthophosphoric acid with phenolic or alcoholic hydroxyl groups:

$$R.O.PO_3^= \rightleftharpoons R.OH + HPO_4^=.$$

The equilibrium lies well over on the side of free phosphate. Studies of the histochemical localization of alkaline phosphatase were made by Robison (1923), who showed that if a section of bone is incubated in a solution containing a phosphoric acid ester and calcium, the phosphate which is split off from the ester by phosphatase is precipitated as calcium phosphate in the vicinity of the enzyme. Considerable advances in our understanding of the mechanism of bone formation were made with the use of this technique. But it is not until comparatively recently that the technique has been developed to provide a close description of the histological distribution of phosphatase, or of its cytological distribution. Takamatsu (1939) and Gömorri (1939) published, almost simultaneously, descriptions of a method for determining the localization of alkaline phosphatase in sections prepared by the usual histological methods. In their procedure a piece of tissue is fixed with 80% alcohol, dehydrated with alcohol, cleared with benzene or cedarwood oil, and embedded in wax at a temperature not exceeding 60° C. Sections are cut from this block with a microtome, mounted in the usual manner on microscope slides, the wax removed with xylol, after which the sections are taken down through alcohol-water mixtures into distilled water. These sections are incubated at 37° C. in a solution containing glycerophosphate and calcium, at *p*H 9·3. Calcium phosphate is deposited in the sections as a result of the enzymatic splitting of glycerophosphate. The sections are treated with cobalt nitrate, washed with distilled water, treated with ammonium sulphide, washed in tap water and mounted in canada balsam. Cobalt phosphate is less soluble than is calcium phosphate; consequently the treatment of a section with cobalt solution changes any calcium phosphate present to cobalt phosphate. Also, cobalt sulphide is less soluble than cobalt phosphate; consequently the treatment of a section with ammonium sulphide converts the cobalt phosphate into cobalt sulphide. Cobalt sulphide is black; consequently a black stain appears in the sections in the positions where initially calcium phosphate had been precipitated following enzymatic hydrolysis of glycerophosphate. A typical result is shown in Pl. 3, fig. 1.

In the papers quoted above, and in numerous publications by other authors, it has been claimed that the histological distribution of the precipitated cobalt sulphide corresponds to the distribution of phosphatase in the sections, and that the site of the precipitate is even an exact guide to the cytological distribution of phosphatase. No proof of these claims has hitherto appeared, though in some cases by experiments on enzymatic activity of fragmented tissues, conducted by the ordinary biochemical techniques for estimating phosphatase activity, it is possible to confirm the qualitative correctness of the results obtained (Fell & Danielli, 1943). If the cytological distribution of phosphatase is in fact revealed by this technique, invaluable information would thereby be made available. But from a cytological point of view no reliance whatever can be placed in the technique until answers can be provided to three questions. These are:

(1) How much phosphatase is destroyed by the experimental procedure; and if there is destruction, does it take place selectively at certain sites, or equally at all sites?

(2) Is calcium phosphate in fact precipitated at the site of enzyme action, or is it precipitated at other sites which have a specific affinity for calcium phosphate?

(3) Is the phosphatase in a fixed preparation in its physiologically normal position?

In this paper answers are presented to these three questions, and the technique has also been subjected to a general examination.

METHODS AND MATERIAL

Most of the work described here was carried out with rat kidney. Occasionally the experiments were repeated with rabbit kidney and sections of healing skin wounds from rats.

Pieces of tissue not more than 3 mm. thick were treated as follows, except where otherwise stated:

fixation was for 2 hr. in 80% alcohol, followed by dehydration in absolute alcohol with either two or three changes; clearing was carried out with cedar-wood oil with two or three changes, and the tissue embedded at 60° C. in wax, m.p. 54° C., for 8 to 24 hr., with two changes of wax. The blocks were kept in the refrigerator until sectioned. Sections were cut at between 2 and 10 μ, flattened with distilled water, dried at room temperature in a desiccator, then taken through xylol (three changes), absolute alcohol (two changes), celloidin (0·1%), and down to distilled water. The sections were incubated at 37° C. in the following mixture:

2% sodium veronal, 20 c.c.
2% sodium β-glycerophosphate, 20 c.c.
2% calcium nitrate, 10 c.c.
Distilled water, 50 c.c.

Control sections were incubated in a similar solution from which the glycerophosphate had been omitted. The sections were incubated for periods varying from 5 min. to 48 hr. They were then washed in 1% calcium nitrate, transferred to 1% cobalt nitrate (2 min.), washed in distilled water (1 min., two changes), treated with ammonium sulphide (1 min.), washed in tap water (5 min.) and then taken up through the alcohols into xylol and mounted in canada balsam as quickly as consistent with efficient dehydration and clearing.

The technique described above was that used when the phosphate released by enzyme activity was to be trapped. Alternatives used instead of glycerophosphate were sodium phenol phosphate and calcium phenolphthalein phosphate. The glycerophosphate and phenol phosphate were the usual samples supplied by British Drug Houses, and the phenolphthalein phosphate was a gift from Prof. E. J. King.

Alternative experiments were also carried out in which a phenol, released from a phenol phosphate by enzymic action, was trapped instead of the phosphate residue by carrying out the enzyme reaction in the presence of a diazonium hydroxide, which precipitated the released phenol as an insoluble dye. Material for these tests was prepared as before, and incubated for between 5 min. and 2 hr. at temperatures between 6 and 37° C. The incubation mixture was made up as follows:

A phenol phosphate, 0·1 g. suspended or dissolved in 30 c.c. distilled water.

2% sodium veronal, 30 c.c.

A diazonium hydroxide solution, 30 c.c.

The diazotized aromatic amines were prepared shortly before use by the methods given by Saunders (1936), kept at about 6° C. until just before use, then brought to *p*H 9·2, with thymol blue as an indicator, after which the veronal and phenol phosphate solutions were added, and the mixture filtered after standing for from 3 to 5 min. at between 6 and 10° C. Control sections were incubated in a similar solution from which the phenol phosphate had been omitted.

The phenol phosphates used were sodium phenol phosphate, calcium phenol phosphate, calcium β-naphthol phosphate and sodium β-naphthol phosphate. Crude calcium β-naphthol phosphate was prepared by the method of Menten, Junge & Green (1944) and purified from excess calcium and free phosphate by washing at *p*H 5·0 with dilute acetic acid followed by distilled water. Sodium β-naphthol phosphate was obtained from this by adjusting the *p*H of an aqueous suspension of the calcium salt to 9·0 with caustic soda, and adding the weighed amount of sodium oxalate required to precipitate the calcium, after which the suspension was kept at 80° C. for 1 hr. with stirring. The β-naphthol phosphate slowly went into solution and concomitantly calcium oxalate was precipitated. The precipitate was filtered off and the sodium β-naphthol phosphate stored as a 5% solution at 0° C.

The amines used were α- and β-naphthylamine, paranitroaniline, α-amino-anthraquinone, and benzidine.

Loss of enzyme activity caused by the histological technique

Under this heading will also be included apparent losses of activity, caused by loss of material from the tissue section at the various stages in the treatment.

Effect of fixation. A comparison was made of the phosphatase activity of rat kidney tissue, (*a*) in the unfixed condition and (*b*) after 2 hr. fixation in 80% alcohol followed by 2 hr. in absolute alcohol and subsequent removal to distilled water. The phosphatase activity was determined by the method given previously (Fell & Danielli, 1943). Also, frozen sections were cut (*a*) from a block of fresh kidney, and (*b*) from the same block after it had been fixed in alcohol as described above. The phosphatase activity of these sections was then compared. The sections were cut at 10 μ. It was concluded that no significant loss of alkaline phosphatase activity occurs as a result of fixation in ethyl alcohol and the cutting of frozen sections.

The effect of embedding, etc. Frozen sections were cut from an alcohol fixed block and incubated with the usual glycerophosphate mixture. The intensity of the reaction in these sections was compared with that developed in sections which had been cut from a block made from the same kidney which had been embedded in paraffin. To facilitate comparison of the activity of the two sets of sections, different sections were incubated for 10, 20, 40, 80, 160 and 320 min. It was estimated that as a result of embedding, etc., up to 75% of the enzyme activity was lost. The distribution of enzyme activity was the same in both sets of sections, i.e. the loss of activity did not appear to be confined to enzymes in any particular position, either histological or cytological. Comparison was also made between blocks which had been embedded in the normal way under aerobic conditions and other blocks which were subjected to all the operations of fixation and embedding *in vacuo*. It was found that more enzyme was lost under anaerobic than under aerobic conditions, but again the loss of enzyme appeared to be fairly evenly distributed over the various sites of activity. It is probable that, under aerobic conditions, glutathione in the tissue is oxidized by atmospheric oxygen, whereas, where oxygen is largely removed, the glutathione is partly present in the reduced state and inhibits the enzyme.

The critical timing of operations. A few preliminary experiments showed that great losses of apparent enzyme activity occurred if some of the steps in the procedure were unduly protracted. The following experiments were therefore performed to ascertain the limits within which the operations could be carried out. First two blocks were taken and fixed in 80% alcohol. One of these was allowed to spend 2 hr. in each of the dehydrating and clearing mixtures and embedded in wax taking 8 hr. at 60° C. The other block was left for 24 hr. in each dehydrating and clearing mixture and embedded for 48 hr. at 60° C. No significant difference was found in the intensity of the phosphatase reaction in sections cut from the two blocks. Secondly, a series of sections was cut from a block prepared in the usual manner and these sections divided into two groups. One group of sections was taken through each step in the procedure as rapidly as possible, with one hour's incubation. Of the second group the sections were taken through all of the steps except one, as quickly as possible, but were left for 24 hr. exposed to the conditions of one step in the procedure; i.e. at 37° C. in air after flattening, or in one of the xylols or alcohols, or in celloidin or distilled water before incubation, or in calcium nitrate, cobalt nitrate, distilled water, ammonium sulphide, tap water or in one of the alcohols or xylols after incubation. Losses of up to 100% of the apparent activity occurred in some cases; for these the effect of a less protracted exposure to the injurious reagent was studied. Table 1 shows (*a*) the duration of each step found convenient in practice, and (*b*) the maximum duration of any given step which has been found to cause no significant loss of activity.

From the table it will appear that some of the steps, especially (11), (13) and (14), require very critical timing. If, for some reason, it becomes necessary to hold up the procedure at stage (13) the addition of a little ammonium sulphide to the tap water will permit the extension of this stage over 24 hr., provided the vessel is covered.

Table 1. *Time limits of the various steps in the procedure of Takamatsu and Gömorri*

Step in procedure	Convenient duration of step	Maximum duration of step found to cause no loss of activity
Steps taken before incubation:		
(1) Fixation	2 hr.	24 hr.
(2) Dehydration (3 changes)	6 hr.	72 hr.
(3) Clearing (3 changes of cedarwood oil)	6–24 hr.	72 hr.
(4) Embedding	8–24 hr.	48 hr.
(5) Drying of sections on to slide at room temperature in desiccator, or at 37° C. in incubator	2–24 hr.	7 days
(6) Removal of wax in xylol	3 min.	24 hr.
(7) In celloidin or alcohols	1 min.	24 hr.
(8) In distilled water	5–60 min.	24 hr.
Steps taken after incubation:		
(9) In calcium nitrate	5 min.	7 days
(10) In cobalt nitrate	2 min.	24 hr.
(11) In distilled water	1 min.	2 min.
(12) In ammonium sulphide	1 min.	24 hr.
(13) In tap water	5–10 min.	30 min.
(14) In alcohols and xylols	3 min.	5 min.

Some experiments have also been tried on the keeping properties of blocks. If stored in the refrigerator the phosphatase activity deteriorates only quite slowly, so that the typical picture may be obtained even after 12 months. But once a block has been cut its phosphatase activity may rapidly deteriorate. The reason for this is unknown.

The general conclusion to be drawn from this examination of the technique, as designed by Gömorri and Takamatsu, is that large apparent losses of enzymic activity may occur, but if the timing given above is adhered to the losses are minimized, and under these conditions the losses from the different cytological sites are roughly in proportion to the original enzyme content of these sites, so that qualitatively no defect in interpretation arises from this cause. Little or no loss of enzymic activity occurs when frozen sections are cut from alcohol fixed blocks, provided the timing given above is adhered to.

The correlation between sites of enzyme activity and sites of calcium phosphate precipitation

No investigation has been made hitherto as to whether calcium phosphate is precipitated in the precise site at which phosphatase is active. Three sets of experiments were made to throw light upon this point. These involved

(1) The precipitation of calcium phosphate in sections by non-enzymic procedures.

(2) Study of the ability of calcium phosphate to diffuse from a site of enzymic activity.

(3) Study of the site of appearance of the alcoholic end of the phosphate ester.

Non-enzymic calcium phosphate precipitation. When calcium nitrate and disodium hydrogen phosphate are mixed on a tissue section on a slide, calcium phosphate is precipitated in the section. In some cases the pattern of precipitated phosphate was similar to that found as the result of enzymic activity. More striking results were obtained by treating sections so as to destroy the enzymic activity and then causing phosphate to be liberated in the sections from a labile phosphate ester by spontaneous hydrolysis. The enzyme was inactivated by heating the sections for 10 min. at 100° C. while almost dry, or by heating for 2 min. at 90° C. in distilled water. A labile phosphate ester was obtained by adding hydrogen peroxide to the normal incubation mixture and then adjusting the pH to 9·3 with ammonia: the glycerophosphate from the incubation mixture is oxidized to a compound which is labile at pH 9·3, so that when the slides are incubated in this mixture at 37° C. phosphate is slowly liberated throughout the incubation mixture. Under these conditions some calcium phosphate is precipitated in all parts of the sections, but there is a strong tendency for a large amount of calcium phosphate to be precipitated at the sites which would be supposed on Gömorri's interpretation of the matter to be sites of phosphatase activity. In kidney sections the nuclei in particular take up calcium phosphate very strongly, though the brush borders of the proximal tubules are often not conspicuously active in this respect. In sections of healing skin wounds calcium phosphate liberated by this procedure is strongly taken up by cell nuclei and also by newly formed collagen: these are also the sites at which phosphatase is supposedly revealed by Gömorri's technique.

It is thus clear that there is a general tendency for sites containing phosphatase as revealed by Takamatsu's and Gömorri's technique to be also sites of strong affinity for calcium phosphate. This, however, is not in itself sufficient to invalidate the technique: it may well be that the sites of enzyme activity do in fact also have a high affinity for phosphate.

The ability of calcium phosphate to diffuse in a section. This was investigated by the following technique. As was indicated in the preceding paragraphs, a section which has been heated loses its phosphatase activity, but, if not heated for too long, retains a strong affinity for calcium phosphate in some parts of the section, notably in the cell nuclei. Such sections either of kidney, spleen or a healing wound were placed upon a slide, and upon these sections was placed a frozen section cut from an alcohol fixed block of kidney. Then the underlying section had a high affinity for phosphatase, but no phosphatase activity, whereas the upper section had the normal enzymic activity and affinity for calcium phosphate. These superimposed sections, which were in close contact with one another, were incubated in the normal incubation mixture for up to 12 hr. It was found that over this period, although there was a very great precipitation of calcium phosphate in the upper section, no calcium phosphate was to be found in the lower section. This means that under the conditions of experiment calcium phosphate cannot diffuse in significant amounts from sites at which phosphate has been liberated to sites having a high affinity for calcium phosphate. If this is so, then it must appear that the calcium phosphate is in fact precipitated at the site of phosphatase activity, and Takamatsu's and Gömorri's technique is thereby shown to be reliable.

In the above experiment, frozen sections were cut from blocks which had been fixed initially in 80% alcohol, then taken into absolute alcohol for 2 hr. before bringing down to water. If on incubation of the section the transfer to absolute alcohol was omitted, then some calcium phosphate appeared to have diffused away from the normal sites of enzyme activity, and even appeared on the glass of the slide, or between the glass and the celloidin. This was probably due not to diffusion of calcium phosphate from the site of enzymic activity, but to the diffusion of phosphatase from an inadequately fixed section. A similar, but much more smeared, appearance is presented after incubation of a frozen section cut from an unfixed block. This ability of phosphatase to diffuse in badly fixed material emphasizes the necessity for fixing small pieces of tissue only, in a relatively large bulk of fixative.

The site of appearance of the alcoholic end of the phosphate ester. Although the inability of calcium phosphate to diffuse from sites of formation of phosphate ion to sites of high affinity for calcium phosphate may be held to prove that the phosphate is precipitated at the actual sites of enzyme activity, yet it appeared desirable to check this conclusion by studying the site of appearance of the alcoholic end of the phosphate ester. No satisfactory technique presented itself for making such observations with glycerophosphate, but suitable results were obtained by changing to the use of phenolphthalein phosphate and of phenol and β-naphthol phosphates.

Phosphatase hydrolyses phenolphthalein phosphate into phosphate ion and phenolphthalein. Phenolphthalein phosphate is colourless, whereas phenolphthalein in alkaline solution is red. Consequently, if phenolphthalein phosphate is substituted for glycerophosphate in the incubation mixture, sites of enzyme activity should become red. Sections were cut as before, and incubated for from 1 to 24 hr. in the phenolphthalein phosphate incubation mixture. Under these conditions the phosphate liberated is precipitated as calcium phosphate, and the phenolphthalein is precipitated in the section mainly as the colourless acid form. The sections were removed from the incubation mixture and nearly, but not quite, dried in a current of air, after which exposure to ammonia gas was used to convert the phenolphthalein into the red alkaline form. The amount of drying is very critical. If the section is too dry, no red colour appears, and if it is too wet the alkaline form of the indicator goes into solution and diffuses from its site of precipitation. It was found that, when the water content of the section was correctly adjusted, the red colour of phenolphthalein appeared in the same sites as had hitherto been found to be sites of phosphate precipitation. Photographs were taken of such sections, after which the sections were treated by the usual procedure for making the phosphate visible, then photographed again. Pl. 3, fig. 2 shows mirror images formed by such photographs. It will be seen that there is a close correspondence between the sites of appearance of phosphate and of phenolphthalein in the brush borders of kidney tubules. A similar correspondence was found at sites of fresh collagen formation in healing rat skin wounds, which are also particularly high in phosphatase.

It is unlikely that the coincidence between the sites of appearance of phenolphthalein and of calcium phosphate is due to the same structure having a high affinity for both compounds. This probability was turned into a certainty by precipitating phenolphthalein in a section from aqueous solution, drying it and exposing it to ammonia, which showed that there was no preferential adsorption on the brush borders of kidney sections. There remained the possibility that calcium phosphate might have a high affinity for phenolphthalein. That this was not so was shown by adding phenolphthalein to a veronal-calcium nitrate solution, followed by several drops of disodium hydrogen phosphate solution; calcium phosphate was at once precipitated: the precipitate was centrifuged down and ammonia added to the precipitate and supernatant fluid. There appeared to be rather less phenolphthalein in the precipitate than in the supernatant fluid, showing that phenolphthalein is not preferentially adsorbed on calcium phosphate. It therefore follows that in the experiment with phenolphthalein phosphate, both pieces of the molecule split by phosphatase activity are precipitated at the same site, and this site is not determined by the peculiar distribution of adsorption affinities in the section. It has therefore been proved that the site of phosphatase in the section is in fact revealed by these techniques.

This use of phenolphthalein phosphate is not satisfactory as a general technique for cytological work. The critical drying of the sections produces a high proportion of failures, and the fact that the intensity of the colour produced by phenolphthalein is small renders the technique useless for the study of sites of low phosphatase activity.

The second method of studying the appearance of the alcoholic end of the phosphate ester was based on the fact that, whereas phenol phosphate does not react with diazonium hydroxides, the phenol liberated by enzymic activity does react, forming a coloured azo dye. Suitable choice of the phenol and of the diazonium hydroxide results in the production of a dye which is insoluble in water, and which is therefore precipitated at the site of enzymic activity. Sections were prepared in the usual manner and incubated in a mixture containing sodium phenol phosphate, sodium veronal and a diazonium hydroxide, made up as described under Methods. The incubation was carried out at 5° C., room temperature or 37° C. The hydrolysis proceeds more slowly at the lower temperatures but the resultant preparation is cleaner, due to less spontaneous decomposition of the diazonium hydroxide. The diazonium hydroxides were prepared from *p*-nitroaniline, α-naphthylamine, β-naphthylamine, and α-aminoanthraquinone. Table 2 summarizes the results obtained. Subsequently a paper by Menten *et al.* (1944) appeared employing this principle, but utilizing β-naphthol phosphate; the experiments were therefore repeated with β-naphthol phosphate and are also summarized in Table 2. Typical results are shown in Pl. 3, figs. 3 and 4.

It was concluded that, where the azo dye which was formed was sufficiently insoluble, it was precipitated almost exclusively in the brush borders of the proximal tubules, thus again showing that this is the main site of alkaline phosphatase activity in kidney tubule sections.

This technique is moderately satisfactory, but probably not as satisfactory as that of Gömorri and Takamatsu in revealing sites of low phosphatase activity. The best combination of substances when this azo dye technique is used is β-naphthol phosphate with α-naphthylamine, β-naphthylamine or benzidine.

The normal physiological position of the enzyme

The studies of Hardy (1899) and Fischer (1899) upon fixation artefacts show that great caution must be exercised before we can accept a structure or a localization of a cellular component as in any way corresponding to structures and localizations existing in the living cell. In some cases it is possible to check observations on fixed material against observations on living material: when dealing with the localizations of enzymes this procedure must usually, as in the present instance, be unavailing. But we need not for this reason despair of the possibility of finding the normal site of the enzyme.

In so far as an enzyme appears in an abnormal site as the result of fixation, the abnormality is produced by the application of unphysiological conditions which have promoted unphysiological aggregation of certain colloidal constituents of the cell. If a structure or a localization of a cell constituent is independent of wide variations in the nature of the fixing agent, then the localization must be a physiological one. But there must be a wide variety in the physico-chemical nature of the fixing agents before such a conclusion can be accepted.

To ascertain which fixatives can be used in the study of alkaline phosphatase, sections which had been cut from an alcohol-fixed block were immersed for 2 hr. in one of the following solutions: 8% formaldehyde, saturated picric acid, saturated magnesium sulphate, saturated ammonium sulphate, saturated calcium chloride, saturated mercuric chloride, 1, 5, 10 and 50% acetic acid, 1% trichloracetic acid, 10% pyridine, acetone, chloroform, anhydrous pyridine. The following were found to destroy the enzymic activity: mercuric chloride, trichloracetic acid, and acetic acid in concentration greater than 1%. Some of the other substances caused a reduction in enzyme activity but not sufficient to preclude their use in fixatives. A study of the actions of a number of oxidizing and reducing agents on the activity of phosphatase in sections has been reported elsewhere (Fell & Danielli, 1943). In view of these results fixatives were made up as indicated in Table 3. Small pieces of rat kidney were placed in these fixatives for 2 hr., and frozen sections cut after bringing the blocks down to water. The table shows that in all cases where the fixation was at all satisfactory from the cytological point of view, the phosphatase activity is restricted to the brush borders of the proximal tubule cells and to cell nuclei. Thus fixatives all gave the same distribution of phosphatase, whether they were acid, neutral, alkaline, polar or non-polar, and pre-precipitation by strong electrolytes did not affect this distribution. The evidence is therefore strongly in favour of the view that the alkaline phosphatase in the mammalian kidney is present almost entirely in the brush borders of the proximal tubule cells and in the cell nuclei.

DISCUSSION

It is of interest that the sites of alkaline phosphatase activity should also be sites promoting the precipitation of calcium phosphate. This fact at first throws suspicion upon the technique, but once it has been shown, as in the present instance, that the two activities are to be found at the same site, then this coincidence must be regarded as adding to the reliability of the technique. The cause of the ability to promote precipitation of calcium phosphate is not clear. In view of the common occurrence of this activity in cell nuclei it was thought that possibly it was due to the phosphate groups of nucleic acid acting as condensation centres. Phosphatase is itself inhibited by phosphate ions, even in low concentration, so that clearly phosphatase has a high affinity for phosphate ion: it is therefore possible that the catalytic centres themselves to some extent promote precipitation of calcium phosphate.

The distribution of phosphatase in cell nuclei is of great interest. It is almost, if not quite, invariably

Table 2. *Summary of results obtained by exposing kidney sections containing active phosphatase to a solution containing a phenol phosphate and a diazotized amine*

The remarks in the table refer to the localization of dye produced by the interaction of the diazonium compound with phenol liberated from the phenol phosphate.

Base used	Depth of general staining of control section	Sodium β-naphthol phosphate	Calcium β-naphthol phosphate	Sodium phenol phosphate	Calcium phenol phosphate
α-amino-anthraquinone in saturated solution	Deep	Not localized	Brush borders	Not localized	Not localized
p-nitro-aniline, 0·2%	Pale	Not localized	In cortex but not restricted to brush borders	Not localized	Not localized
Benzidine, 0·2%	Moderate	Brush borders	Brush borders	Not localized	Not localized
α-naphthylamine	Pale	Brush borders	Brush borders	Brush borders	Brush borders
β-naphthylamine	Pale	Brush borders	Brush borders	Brush borders	Brush borders

found in the nuclei of mammalian tissues, sometimes in very high concentration. Its function in these nuclei is unknown, though the possibility that it is concerned in the metabolism of nucleic acid and the nucleotides at once springs to mind. More detailed studies of the distribution of this enzyme in cell nuclei, and a study of its occurrence in other phyla, are clearly called for. It is of interest that McFarlane & Salaman (1938), McFarlane & Dolby (1940), and Hoagland, Ward, Smadel & Rivers (1944) find that alkaline phosphatase is a component of vaccinia virus, though they have not been able to show whether it is an invariable component of the virus, or whether it is adsorbed on to the virus from the cellular debris from whence the virus is extracted.

Table 3. *The results obtained with various fixatives*

Fixative	Shrinkage	Fixation	Enzyme activity
None	o	—	Mainly in cortical tubules; smeared appearance
80% ethyl alcohol	+	Fair	In proximal brush borders and in cell nuclei
Formaldehyde 4% + sodium chloride 1%	o	Good	,, ,, ,, ,, ,,
Acetone	+ +	Fair	,, ,, ,, ,, ,,
Pyridine	+ +	Good	,, ,, ,, ,, ,,
70% pyridine	+	Good	,, ,, ,, ,, ,,
25% pyridine in 80% alcohol	o	Good	,, ,, ,, ,, ,,
1% calcium chloride in 70% pyridine	+	Good	,, ,, ,, ,, ,,
Formaldehyde 4% + calcium chloride 1%	+	Good	,, ,, ,, ,, ,,
Saturated picric 75% + formaldehyde 4%	o	Fair	Enzyme destroyed
Ethyl alcohol 65% + chloroform 35%	o	Poor	Enzyme spread all over proximal tubules
Pyridine 70% + formaldehyde 4%	=	Very poor	Enzyme spread over whole section
Pyridine 20% + ethyl alcohol 70% + 4% formaldehyde	o	Very good	In proximal brush borders and in cell nuclei
Alcohol 60% + chloroform 30% + 4% formaldehyde	o	Poor	In proximal tubules and cell nuclei, and in some places diffused into cytoplasm
Formaldehyde 4% + acetic acid 1%	–	Very good	In proximal tubules and in cell nuclei
Formaldehyde 4% + acetic acid 5%	o	Good	Enzyme destroyed
Ethyl alcohol 80% + acetic acid 1%	=	Fair	In proximal tubules and in cell nuclei
Pyridine 25% + saturated picric 75%	o	Fair	,, ,, ,, ,,
Saturated ammonium sulphate solution followed by 80% alcohol	+	Fair	,, ,, ,, ,,
Saturated calcium chloride solution followed by 80% alcohol	+ +	Poor	,, ,, ,, ,,
Saturated magnesium sulphate solution followed by 80% alcohol	+ +	Fair	,, ,, ,, ,,

+ + gross shrinkage, + some shrinkage, o no change, – some swelling, = gross swelling.

Apart from its occurrence in nuclei and brush borders, alkaline phosphatase seldom appears in the cytoplasm except in rapidly regenerating tissue such as is found in healing wounds and in tissue cultures, where it is likely that its occurrence is connected with the formation of fibrous protein. The formation of collagen fibres is very closely associated with the appearance of alkaline phosphatase on the fibres and in the connective tissue cells forming the fibres: under conditions in which phosphatase does not appear in the cells and on the fibres, only precollagen (argyrophile) fibres are formed (Fell & Danielli, 1943; Danielli, Kodicek & Fell, 1945). The formation of fibrous keratin, in hair follicles, is also associated with intense phosphatase activity, both in the follicle cells and on the newly formed keratin. Apart from these instances, alkaline phosphatase is usually found only in extracellular sites, as in blood, bone, cartilage and in the digestive tract. The cytoplasmic conditions of pH and the occurrence of reduced glutathione in the cytoplasm are both calculated to reduce alkaline phosphatase activity to negligible proportions, whereas, in the extracellular sites, inhibition of the enzyme by SH groups is eliminated. It therefore seems likely that, apart from its possible activity in cell nuclei, alkaline phosphatase has a significant activity only in extracellular sites; its occasional occurrence in intracellular sites does not mean that it is active in these sites, but more probably that the enzyme is on its way to the extracellular sites where its activity will be displayed. The occurrence of active alkaline phosphatase in the cytoplasm would be surprising, since its activity would tend to disrupt many fundamental cellular processes, including the metabolism of glucose and all processes involving phosphorylated nucleosides.

In brief, whereas alkaline phosphatase may well be active in the cell nuclei, and certainly is active in extracellular sites, the conditions of pH and the SH concentration prevailing in cytoplasm are highly unfavourable to its activity. In view of this the position of phosphatase associated with the brush borders of the proximal convoluted tubules of the kidney must be reconsidered. It was suggested by

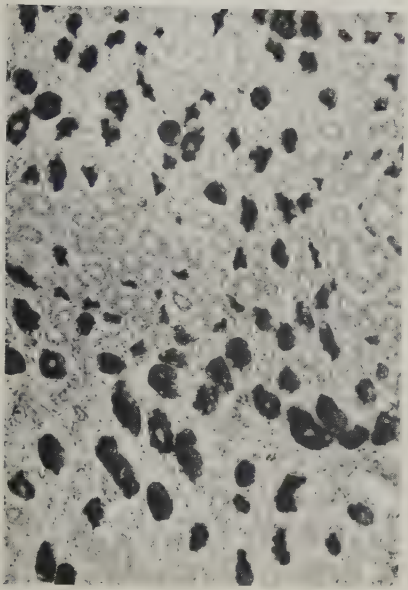

Fig. 1

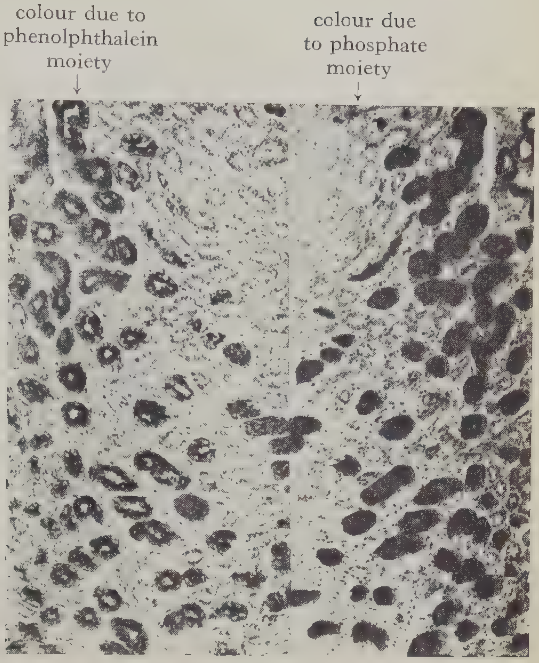
colour due to phenolphthalein moiety
colour due to phosphate moiety

Fig. 2

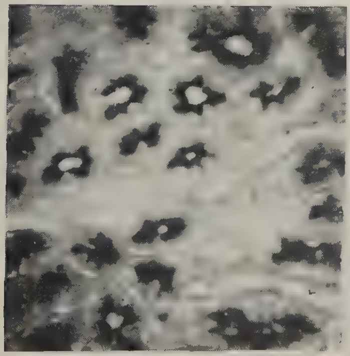

Fig. 3

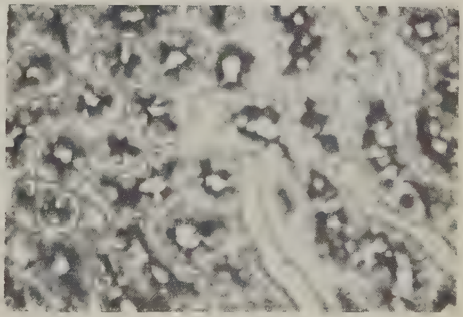

Fig. 4

DANIELLI—CYTOLOGICAL POSITION OF ALKALINE PHOSPHATASE

Gömorri that this enzyme played a part in the phosphorylative reabsorption of glucose from the glomerular filtrate. It is certainly true that the tubular region concerned with the reabsorption of glucose is also that containing the high concentration of phosphatase. But if the enzyme is in fact concerned in phosphorylation cycles it is bound to be intracytoplasmic, and therefore inactive! It seems highly improbable that a high concentration of enzyme would be produced at a site where it can have no activity. I therefore suggest that this enzyme in the brush borders is on the outside of the cells and is concerned, not in phosphorylation cycles, but in the breakdown of phosphate esters present in the glomerular filtrate. Phosphate esters in the filtrate should not be readily reabsorbed, since they penetrate cell membranes very slowly. But if they are broken down into phosphate ion and an alcohol residue, such as glycerol, or glucose, then reabsorption may be relatively rapid. The view suggested here, therefore, is that the phosphatase of the brush borders of the kidney tubules plays an important role in the economy of phosphate which would otherwise be lost in the urine, but does not directly participate in reabsorptive phosphorylation cycles.

SUMMARY

1. It has been proved that the technique of Takamatsu and of Gömorri indicates the cytological sites of alkaline phosphatase activity.

2. The critical time limits for the various operations in the technique have been determined.

3. Several alternative techniques are given for demonstrating phosphatase activity on the cytological level.

4. A selection of fixatives is given which do not produce severe destruction of alkaline phosphatase activity.

5. It has been shown that alkaline phosphatase in fixed preparations is found in sites which are those in which it occurs under physiological conditions.

6. It is suggested that the function of the phosphatase found in the brush borders of the proximal tubules of the kidney is to hydrolyse phosphate esters in the glomerular filtrate, and thereby permit the reabsorption of the component molecules by tubular processes.

I am deeply indebted to Dr H. B. Fell for her general encouragement, and for confirming some of the observations recorded in this paper. Prof. E. J. King very kindly presented me with a specimen of phenolphthalein phosphate, and to Prof. J. Gray and to Prof. A. C. Chibnall I owe thanks for their criticisms of the manuscript. Mr V. Norfield kindly undertook some of the photography.

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EXPLANATION OF PLATE

Fig. 1. Rat kidney by method of Takamatsu and Gömorri. Fixative 80% alcohol. Mounted in balsam. Enzyme in proximal tubule cells and in nuclei.

Fig. 2. Mirror images of rat kidney section. Fixative 80% alcohol. Right, phosphate deposit, mounted in balsam. Left, phenolphthalein deposit, mounted in water.

Fig. 3. Rat kidney. Fixative 80% alcohol. Photograph after use of azo technique (Na β-naphthol phosphate and α-naphthylamine). Mounted in glycerol. Blue filter.

Fig. 4. Rat kidney. Fixative 80% alcohol. Photograph after use of azo technique (Ca β-naphthol phosphate and benzidine). Mounted in glycerol. Green filter.

THE GROWTH OF BROWN TROUT (*SALMO TRUTTA* LINN.)

I. FACTORS INFLUENCING THE GROWTH OF TROUT FRY

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(*Received* 1 *October* 1945)

(With Five Text-figures)

INTRODUCTION

It has long been known that brown trout (*Salmo trutta* Linn.) grow to different sizes in different waters, and there has been much speculation concerning the factors determining these differences. Dahl (1918–19) suggested that the food supply and the degree of crowding were important; also that small, slowly growing trout were derived from smaller ova than those growing more rapidly. Southern (1932, 1935) demonstrated that, in Ireland, the rapidly growing fish are found in hard or alkaline waters, while those growing slowly live in soft, acid waters. This correlation has been upheld by the work of Frost (1939, 1945), Went & Frost (1942) and Went (1943) for Ireland, and by that of Raymond (1938) and Swynnerton & Worthington (1939) for Great Britain, though exceptions were reported in the *Salmon and Freshwater Fisheries Report* for 1936.

The greatest differences between the length and weight increments of trout living in hard and soft waters occur during the fourth year of life, but the annual specific growth rates show the most striking differences during the first year. After this, the average specific growth rates may be very similar in the two types of water although the fish are of markedly different sizes. They begin to spawn at an earlier age in some waters, and fish which spawn have a lower average specific growth rate for that year than fish of the same age which do not. Thus, the size attained by trout in different waters depends on (1) their rate of growth during the first year of life, (2) the age at which they begin to spawn, and (3) their average length of life. In soft waters, trout grow slowly during the first year, begin to spawn when 3 or 4 years old, and seldom live more than 5 years; in hard waters, they generally have a high specific growth rate during the first year, begin to spawn when older and may live for 12 years or longer.

The effect of environmental factors on trout growth is, therefore, of special interest during the first year of life, since there must be a factor or factors which inhibit growth in the soft waters during this year. A number of observations on trout growth are available from experimental hatcheries (McCay & Tunison, 1935, 1937) and from laboratories (Pentelow, 1939; Wingfield, 1940), but they concern fish more than 8 months old. It is generally admitted that a number of environmental factors may affect trout growth, but it has so far been impossible to correlate differences in growth rate with any particular factor, since several environmental factors have varied simultaneously without being measured accurately.

The present experiments were designed as an investigation of the rate of growth of individual trout in environments where as many factors as possible were controlled. Temperature, amount and intensity of illumination, chemical composition and aeration of the water, rate of water flow and quality of food supply were maintained as constant as possible, and the fish were allowed to eat as much as they would. Under these conditions it was hoped that the growth rates of the fish would vary in a definite and orderly way. Any changes in growth rate common to all the individuals should be the result of more or less intrinsic physiological factors, such as periodic secretion of endocrine organs. Any differences in growth rate exhibited by individuals in the same group might be ascribed to genetical differences or to the effect of the group on the individuals of which it is composed. When one environmental factor is varied and the others maintained constant, changes in the growth rate, compared with control fish, should indicate the effect of the varying factor.

The present paper is concerned with the growth of trout fry during the 8 months following the beginning of feeding. Although it is recognized that the conclusions derived from this study of the effect of genetical constitution, alevin weight, crowding and intraspecific relationships on specific growth rate are based on limited data, they are presented because they indicate some interesting possibilities.

APPARATUS AND TECHNIQUE

Trout ova were obtained from The Midland Fishery, Nailsworth, Gloucestershire. On 10 December 1943, Mr Stevens stripped two 4-year-old brown trout, females A and B, of their ova, and fertilized each lot with milt from the same 4-year-old male fish. The fertilized ova were incubated at the hatchery in spring water at a temperature of about 10° C. The two sets of 'eyed ova' were sent to Cambridge by rail on 14 January 1944, and were there kept in troughs supplied with tap water at about 13° C. The majority of the ova obtained from female A had hatched by 21 January, while those from female B began to hatch on 20 January, and the majority had emerged by 24 January.

All the ova were dried on filter paper and weighed individually with a torsion balance before they hatched, and the alevins were then reared in hatchery trays in the dark. They were dried with filter paper and weighed individually when the yolk sac had nearly been absorbed and were then sorted into groups to be used for experiments. The fry descended from female A began to feed on 19th February, and those descended from female B on the 26th. The weight distributions of the two populations of ova and alevins are shown in Fig. 1. In each case the alevins had a wider dispersion of weight and average weights which were 11–12 % higher than those of the ova from which they were derived (Table 1). The alevins descended from female A were used in the majority of experiments rather than those descended from female B, because the former were more numerous and their weight distribution curve approximated more closely to a normal curve.

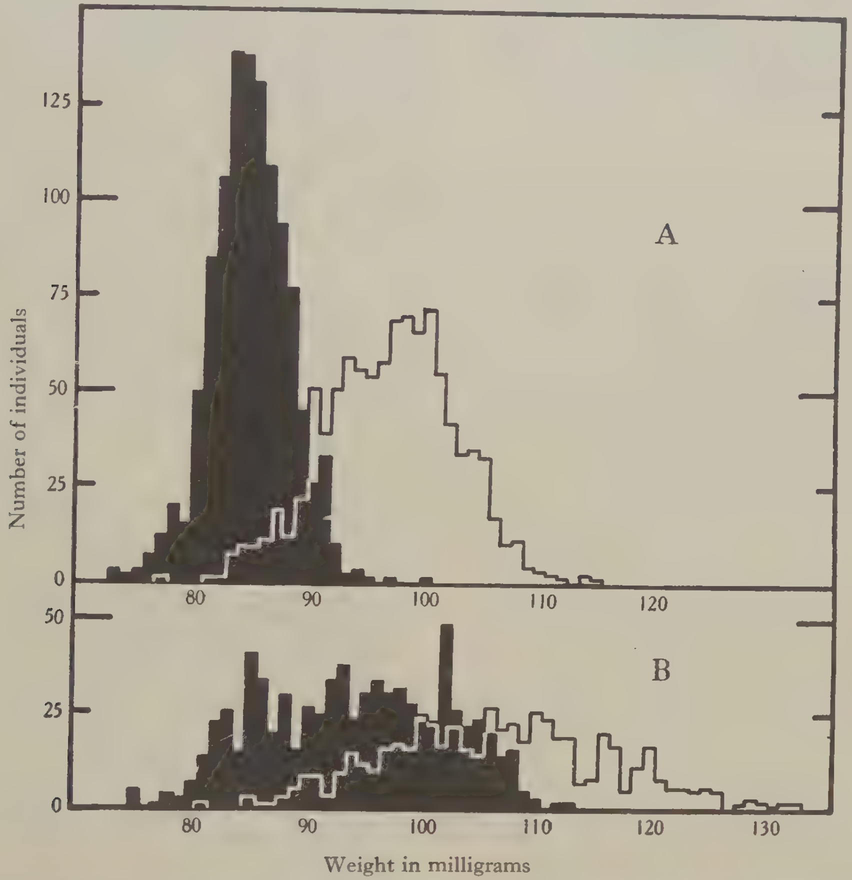

Fig. 1. Histograms to show the distribution of weight among the populations of ova and alevins. A, descended from female A. B, descended from female B. Solid black: ova. White: alevins.

The alevins were grown in a constant-temperature room which was set at 11·5° C. This temperature was chosen because it lies within the optimum range for trout growth postulated by Pentelow (1939) and Wingfield (1940). The fish were kept in glass battery jars each containing 7 l. of Cambridge tap water, an analysis of which is given in Table 2. The water supply came from a constant-level tank so that it was under constant pressure. On entering the room the water flowed through a coil of metal tubing in the air and then through another coil immersed in standing water. This reduced its temperature to that of the room, and it seldom varied more than 0·2° from 11·5° C.

Each battery jar had its own water supply which sprayed through a jet and overflowed through a constant-level siphon into a gutter which ran to waste. The water in the jars was stirred continuously with compressed air. The room was free from daylight; the fish were illuminated by 40 W. electric bulbs suspended 36 cm. above the water surface and so arranged that there was one bulb for each 20 sq.cm. of surface area. The lights were controlled by a clockwork time switch so that the fish were illuminated for 12 hr. and in darkness for 12 hr. each day.

Table 1. *Summary of data about ova and alevins*

	Derived from female A	Derived from female B
Date of fertilization	10 Dec. 1943	10 Dec. 1943
Time of hatching as days after fertilization	40–42	41–45
Time of beginning of feeding as days after fertilization	71	78
No. of ova weighed	1093	761
No. of ova died before hatching	71	142
No. of alevins died before weighing	17	33
No. of abnormalities	12	27
No. of alevins weighed	993	559
Average weight of ova (mg.)	84·33	94·94
Average weight of alevins (mg.)	94·3	105·4
Percentage increase in average weight	11·9	11·2

Table 2. *An analysis of Cambridge tap water* (*on* 3 *March* 1944)

(By courtesy of the Cambridge University and Town Waterworks Co.)

Chemical composition as parts per million

Total dried solids: 305.
Chlorides: 15.
Alkali reserve (as calcium carbonate): 200.
Total hardness: 130.
(all temporary, no permanent hardness).
Calcium as Ca: 59·0.
Magnesium as Mg: 1·6.
Nitrogen as nitrates: 6·4.
Nitrogen as nitrites: 0.
Ammonia: 0.
Albuminoid ammonia: 0.
Iron as Fe: 0·07.
No other metals present.
Oxygen absorbed in 4 hr. at 27° C.: 0.
Free chlorine reaction: absent.
*p*H: 7·3.

'Very high standard of organic and bacterial purity.'

The fry were fed on finely minced raw liver and were always given more than they would eat. The liver was dropped into the water, and the fry took it readily as it sank slowly to the bottom. At first they were fed twice daily, but after 3 months of feeding they were fed only once daily. The remains of food and faeces were siphoned out of the tanks every day, but there was no attempt to measure the actual food consumption of the fry.

At intervals all the fry in each jar were removed; each fish was placed in 2·5 % urethane solution until it rolled over on to its side, and it was then dried on filter paper and weighed. Those weighing less than 350 mg. were weighed with a torsion balance to the nearest milligram, and heavier fish were weighed on a chemical balance to the nearest 5 mg. After weighing, the fry were placed in a shallow dish of water with ice floating in it, and they generally recovered within 2 min. There were very few deaths as a result of weighing; these were caused by the use of too strong a solution of urethane.

THE GROWTH OF INDIVIDUAL TROUT FRY IN CONSTANT ENVIRONMENTAL CONDITIONS

Some of the alevins descended from female A were sorted into five batches of fifty individuals, each group with a limited range of individual weight, but covering between them the total range in weight of this family. Others were grown in similar environmental conditions but with different numbers of individuals in each tank. Two sets of fifty alevins descended from female B were also grown in the same environmental conditions. One of these groups included a range in weight which was the same as that of the majority of the alevins descended from female A, while the other consisted of individuals of greater weight.

At the beginning of the experiments some fry began to feed before the others and some never fed at all. The latter died within the first 3 weeks and others died at intervals later, but over 50 % of the individuals in each group survived for more than 8 months. The record for each tank showed a progressive decrease in the number of surviving fry, but only the smallest individuals died. Certain individuals were marked, and these remained in approximately the same numerical position if the weights of the fish were tabulated in descending order of size after each weighing.

It was decided not to use the mean weights for the groups in comparing the performance of fry in different tanks, since the weight distributions were not normal. The median value depended on the number of fry surviving at any date and would give no useful data for individual growth, though the medians and interquartile ranges indicated the dispersion of weight within a group. In order to estimate the growth of individuals, it has been assumed that each individual remained in the same position in order of descending weight for the group, so that, for instance, the values that were first, fifth, tenth, fifteenth and twentieth in successive weight records showed the change in weight of individuals occupying these positions in the order of size. With this assumption, individual specific growth rates were calculated from the formula

$$G = \frac{\log_e Y_T - \log_e Y_t}{T - t} \times 100,$$

where G = specific (or incremental, geometric, instantaneous or multiplicative) growth rate, Y_T = weight at time T, Y_t = weight at time t, and T is later than t. The time was expressed as weeks, so that the specific growth rate is expressed as percentage weight per week.

When the growth records of the five groups of fifty fry descended from female A were compared, it was clear that the individuals did not all grow at the same specific rate, although they had the same parents and were living in the same environmental conditions. The dispersion of individual weights increased with time (Table 3), and individual specific growth rates were generally higher for the fish which grew most rapidly during the first period than for the smaller fry (Table 4). Thus, for the first few months, the order of descending weight was also an order of decreasing specific growth rate for the group. All the fish showed decrease in their specific growth rates as they grew older, and the difference in rate between the members of a group decreased with time.

The effect of 'grading' on individual specific growth rates

The difference between the rates of growth of large and small individuals in the same group might be the result of two different causes: (1) some fry might be hereditarily incapable of as high a specific growth rate as others, since the individuals were not necessarily identical genetically although they were the offspring of the same parents and these came from an inbred hatchery stock; (2) the differences might result from some fish beginning to feed earlier than others and thus acquiring an initial advantage in size. Mutual reaction between fish of different sizes might then result in the larger fish growing faster and the smaller ones being retarded. Since the fish were allowed as much food as they would eat, competition for a limited food supply cannot be the factor involved.

In order to test this second hypothesis, two groups of fifty alevins descended from female A were grown under similar environmental conditions for 8 weeks, and all the fish in one group were marked by amputation of the adipose fin. The two groups were then combined, and the individuals were graded into equal numbers of 'large' and 'small' fry, i.e. those weighing more and less than 350 mg. respectively. These two groups were grown under the same environmental conditions as the original groups, and were weighed at intervals so that specific growth rates might be calculated.

Table 3. *Dispersion of individual weights within groups of trout fry*

M, median value. IqR, interquartile range.

No. of days after beginning of feeding	Weight in mg.					
	Small		Medium		Large	
	M	IqR	M	IqR	M	IqR
0	86	5	95	0	104	5
21	132	38	160	44	166	59
35	158	57	247	102	232	101
56	253	140	280	98	310	245
96	380	264	482	188	531	452
119	399	283	590	328	616	534
140	433	377	651	445	684	593
163	520	433	761	486	945	900
183	720	586	910	803	1470	1540
206	795	755	878	1094	1370	1202
231	1120	1045	1305	1620	2180	2375

Table 4. *Mean specific growth rates (as percentage weight per week) of individual trout fry descended from female A*

The alevins all weighed 95 mg. before they began to feed.

Position in size order	Fish no.					
	1	5	10	15	20	25
Specific growth rate during weeks						
0– 3	26·0	23·3	21·7	20·9	20·0	16·5
3– 8	16·8	13·7	12·0	9·2	8·1	7·9
8–14	11·6	11·5	8·5	9·1	9·1	7·4
14–20	4·5	4·3	6·9	6·3	2·7	3·5
20–26	9·5	8·6	6·7	6·5	3·9	3·5
26–33	10·6	6·3	5·7	2·5	−0·9	—

Within each group, the larger individuals grew faster than the smaller fish, but the range in specific growth rate was approximately the same among the 'large' and 'small' fry (Fig. 2 and Table 5). The largest of the group of 'small' fry actually grew at a higher specific growth rate than the largest of the group of 'large' fry. The smallest fry of the 'large' group grew at a lower specific rate than the largest of the 'small' group, although before grading they had grown at nearly equal rates.

After 7 months, the survivors of the two groups were transferred to another tank, and all the 'large' group were marked by amputation of the right, and all the 'small' group by amputation of the left pelvic fin. At this date, the range of weight in the two groups was nearly the same—from 770 to 8390 mg. in the 'large' and from 740 to 8150 mg. in the 'small' group. Of the fifteen largest fish, ten came from the 'large' and five from the 'small' group, while of the fifteen smallest fish, ten came from the 'small' and five from the 'large' group.

In the two months following this regrouping, the five largest fish of the 'large' set, now fish 1, 3, 4, 5 and 6 of the mixed group, showed little change in average specific growth rate; the next five largest, now fish 7, 8, 11, 12 and 15, showed slight reduction in rate; but the five largest of the 'small' fish, now in positions 2, 9, 10, 13 and 14 of the mixed group, grew at a markedly lower average specific rate (Table 6). Fish with lower positions in the size orders also showed the effect of regrouping. Those in positions 7–10 inclusive in the 'small' group and those in positions 16–19 in the 'large' group together filled positions 22–29 inclusive in the mixed group. After regrouping, the 'small' fish grew at a reduced and the 'large' fish at an increased average specific rate so that the rates were nearly equal, although before regrouping the 'small' fish had been growing faster.

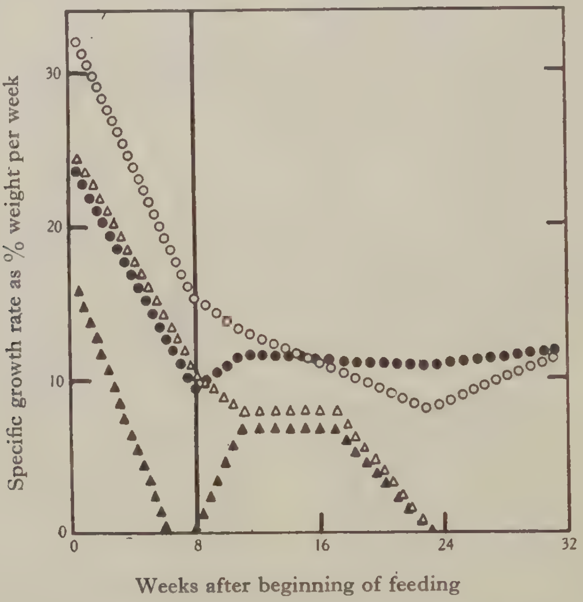

Fig. 2. The specific growth rates of four individual trout fry, all grown in the same environmental conditions. ○ This fish was the largest individual in its group throughout the experiment. Before week 8, the group consisted of large and small fry; after week 8, of large fry only. △ This fish was the twentieth in the order of decreasing weight for its group during the first 8 weeks. It then became the smallest individual in a group consisting of large fry only. ● This fish was the twenty-first in the order of decreasing weight for its group during the first 8 weeks. It then became the largest individual in a group consisting of small fry only. ▲ This fish was the smallest individual in its group throughout the experiment. Before week 8, the group consisted of large and small fry; after week 8, of small fry only.

These observations all imply that the specific growth rate of an individual in a group of trout fry depends on its size relative to that of the other individuals, the larger fry growing faster than smaller ones. If the larger fry are removed, the smaller ones grow at an increased rate, and if larger fry are added, the smaller ones show a decrease in specific growth rate. There is no evidence that genetical factors play any important part in this difference in specific growth rate between individuals of the same family living in the same environment, though they may determine the readiness with which alevins begin to feed, and hence their initial specific growth rate.

Table 5. *Comparison of the specific growth rates (as percentage weight per week) of large and small fry of the same age and with the same positions in the orders of size for their groups*

Age as weeks after beginning of feeding	Position in order of size	Initial weight in mg.	Specific growth rate
8–14	1	360	11·3
		589	13·3
	5	338	10·2
		496	11·7
	10	317	9·1
		476	9·9
	15	294	10·0
		450	10·0
	20	276	9·6
		421	9·6
	25	244	8·6
		402	8·6
14–20	1	720	11·1
		1310	10·7
	5	620	8·5
		1002	10·5
	10	550	8·3
		862	9·7
	15	491	8·9
		823	8·9
	20	460	9·9
		749	9·9
	25	420	9·3
		675	9·3
20–26	1	1402	10·7
		2500	7·9
	5	1032	5·9
		1880	2·1
	10	904	3·4
		1550	3·2
	15	812	2·6
		1410	2·8
	20	673	1·5
		1355	0·3
26–33	1	2670	11·6
		4005	10·6
	5	1480	9·2
		2146	10·2
	10	1110	5·6
		1880	5·2
	15	950	2·1
		1675	4·2
	20	740	1·5
		1390	1·5

The effect of crowding on individual specific growth rates

The growth rate of an individual in a group of trout fry might depend either on the total number of fish that are of larger size, or on the proportion of fish that are larger and smaller. Comparison of the growth of individuals in groups consisting of different numbers should make it possible to distinguish between these alternatives. Such groups, starting with 25, 50, 80, 100 and 150 alevins descended from female A, were grown in similar environmental conditions for 5 months after they began to feed. The mortality was consistently highest in the most crowded tank, but there was no marked difference in percentage survival between the four less crowded tanks. In every case, the fish which died were small and thin, but they did not appear to suffer from any definite disease.

In Fig. 3 the specific growth rates of the twenty largest fry in each group are compared. The values for the three larger, more crowded groups vary in the same way, those for the eighty alevin group being highest and those for the 150 alevin group lowest. If the values for the largest fish are excepted, the difference in growth rate among these fish is greater in the smaller, less crowded groups, especially in that started with twenty-five alevins.

Fig. 4 shows the specific growth rates of individuals occupying positions $n/20$, $2n/20$, $3n/20$, $4n/20$, $5n/20$, $6n/20$, $7n/20$, $8n/20$, $9n/20$ and $10n/20$ in the size orders, where n is the original number of alevins in the group. The values for the three less crowded, smaller groups are very similar, while those for the two more crowded, larger groups cover a greater range of specific growth rate.

The fish in the tank started with eighty alevins grew at the highest specific rates in both cases, so this size of group and degree of crowding appear to be optimal for the environmental conditions used. Compared with this group, the specific growth rates of individuals in larger, more crowded groups depended on the number of fish of larger size, while individuals in smaller, less crowded groups grew at rates depending on the proportion of fish which were larger and smaller. Competition for a limited food supply can have had no effect, at least in the less crowded groups. The lower growth rates of smaller fry can be attributed to some 'social organization' within the group, those which began to feed earlier attaining a higher position in the hierarchy than the others and therefore continuing to grow at higher specific rates.

There was an optimum population density for which the yield of fish weight was maximum. In Fig. 5 the total weight of fish in each experimental tank is shown at intervals of 10 weeks. After 5 months the tank which was started with eighty alevins contained the greatest weight of fish, and the values decrease for degrees of crowding above and below this optimum.

The change in specific growth rate with time

The specific growth rates of most animals decrease with increasing age and size, and this occurs also with trout fry. The specific rate is highest during the first 3 weeks after the beginning of feeding, and the average rates decrease as the fish grow older (Table 4), though there is some variation from week to week. Within each group the larger fish grew faster, but Table 5 shows that fish of the same age, but of very different sizes, grew at approximately the same rates if they occupied the same positions in the size orders of their groups. Thus, for trout fry, age is a significant factor determining the negative acceleration of specific growth rate with time, and there is no correlation between body weight and specific growth rate.

THE EFFECT OF ALEVIN WEIGHT ON THE SPECIFIC GROWTH RATE OF FRY

During the period between the hatching of the ova and the beginning of feeding, the alevins increased in

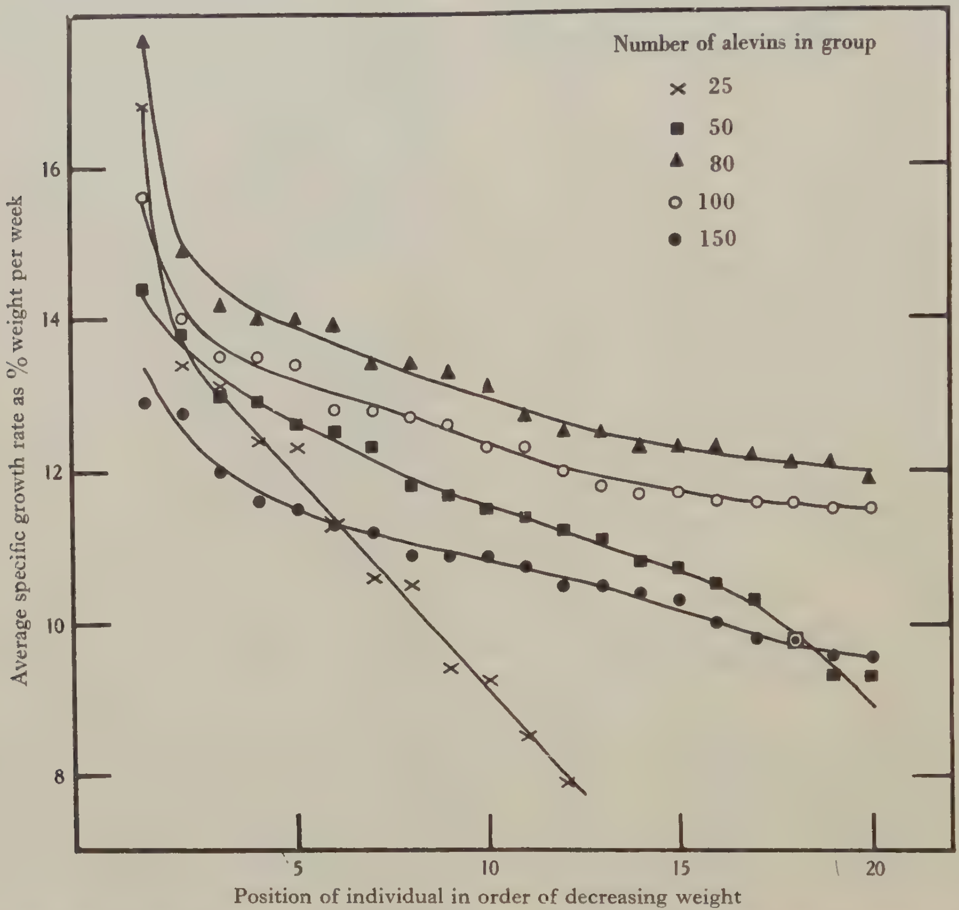

Fig. 3. Mean specific growth rates for 20 weeks after the beginning of feeding of the twenty largest individuals in groups containing different numbers of individuals.

Table 6. *Comparison of the average specific growth rates of large and small fry for 10 weeks before and after regrouping*

Group	Positions in size order		Average specific growth rates (as % weight per week)	
	Before regrouping	After regrouping	Before regrouping	After regrouping
Large	1, 2, 3, 4, 5	1, 3, 4, 5, 6	8·9	9·0
Small	1, 2, 3, 4, 5	2, 9, 10, 13, 14	10·2	8·5
Large	6, 7, 8, 9, 10	7, 8, 11, 12, 15	6·5	6·0
Large	16, 17, 18, 19	22, 23, 27, 29	3·3	4·5
Small	7, 8, 9, 10	24, 25, 26, 28	6·3	5·0

weight by absorption of water (Gray, 1926), but the larger alevins were those which had hatched from larger ova. The three groups of fifty alevins descended from female A and the two groups of fifty alevins descended from female B, each group with a limited range of weight at the beginning of the experiments, thus represented a range in ovum weight.

The two groups of alevins descended from female B did not include either the largest or the smallest alevins of their family, but differed from each other more markedly in weight than did any of those obtained from female A. The smaller alevins grew initially at a higher specific rate, so that the larger fry of the two groups were of approximately the same weight after 2 months. After this the fry derived from the larger alevins grew slightly faster, but at the end of 8 months, individuals in the same positions in the size orders did not differ markedly in weight (Table 7).

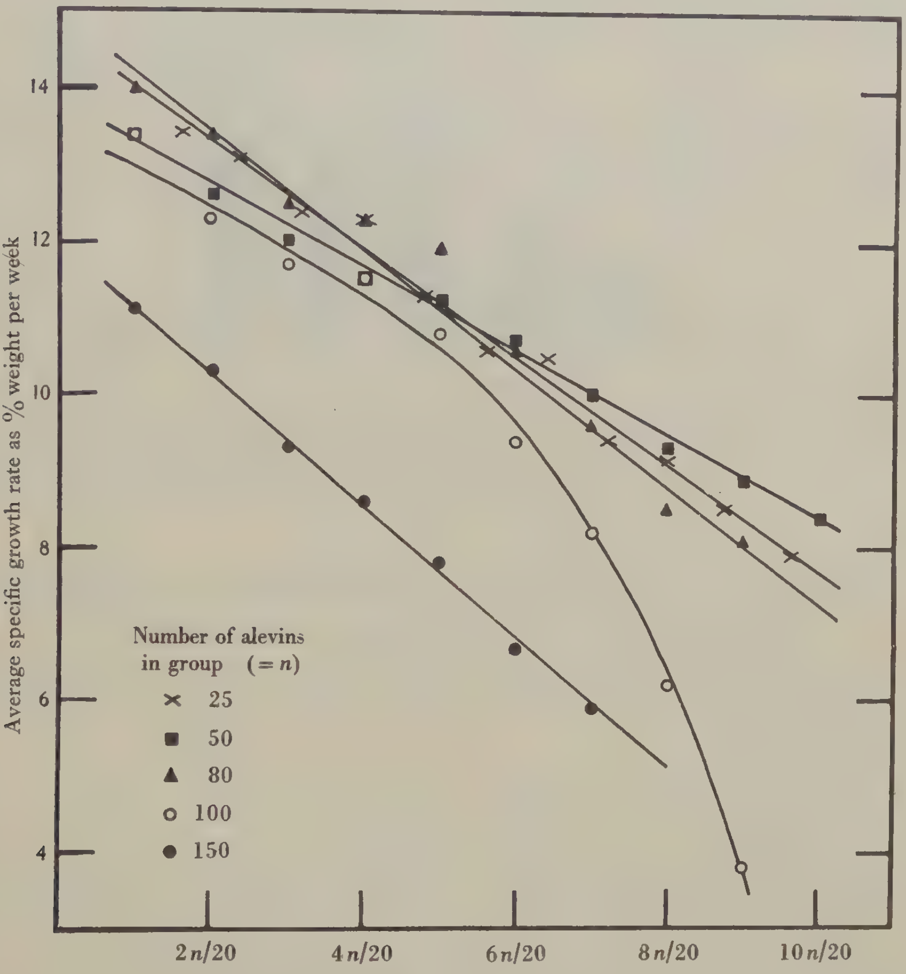

Fig. 4. Mean specific growth rates for 20 weeks after the beginning of feeding of individuals occupying positions $n/20$, $2n/20$, $3n/20$, $4n/20$, $5n/20$, $6n/20$, $7n/20$, $8n/20$, $9n/20$ and $10n/20$ (where n = number of alevins) in the size orders of groups containing different numbers of alevins.

During the first 8 months there was little difference between the average specific growth rates of fry derived from alevins of different weight, descended from female A, if individuals in the same positions in the size orders for their groups are compared (Table 7). Since, however, the alevins were of different initial weights, the larger ones grew into slightly larger fry than the smaller ones (Table 3).

The groups of fry descended from female A and from female B thus showed different effects of alevin weight on early growth, but the range of specific growth rate within each group was greater than the

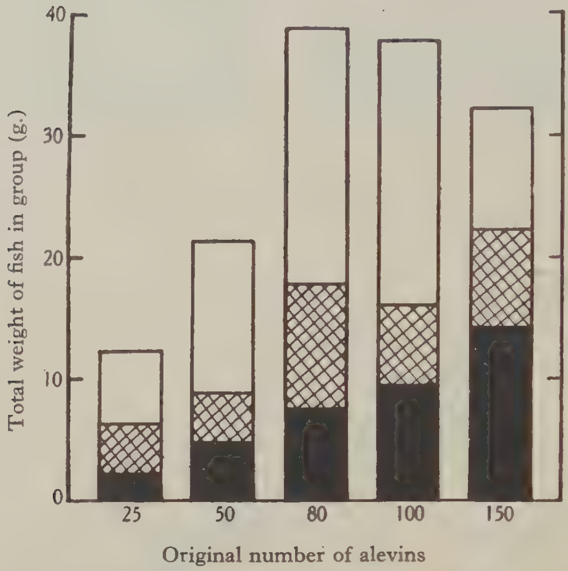

Fig. 5. Histograms showing the total weight of fish in groups of trout fry which were started with different numbers of alevins in equal volumes of water. Solid black: weight at the beginning of feeding. Cross-hatched: weight added during weeks 0–10 after the beginning of feeding. White: weight added during weeks 10–20 after the beginning of feeding.

Table 7. *Comparison of the growth of fry derived from alevins of different weights*

The mean specific growth rates are expressed as percentage weight per week. The weights of the fry are given in milligrams.

Position in size order ...	1	5	10	15	20
Fry descended from female A:					
Mean growth rate (weeks 0–33):					
Alevin weight 80–89 mg.	12·78	9·94	8·58	7·81	6·91
95 mg.	12·20	9·98	9·03	7·10	6·45
101–110 mg.	12·21	10·39	9·20	7·36	6·43
Weight at end of week 33:					
Alevin weight 80–89 mg.	5770	2265	1445	1120	830
95 mg.	5320	2550	1870	990	800
101–110 mg.	5900	3233	2180	1200	875
Fry descended from female B:					
Mean growth rate (weeks 0–8):					
Alevin weight 90–100 mg.	17·7	15·4	14·2	13·1	11·1
120–130 mg.	15·4	11·8	11·1	9·9	9·3
Weight at end of week 8:					
Alevin weight 90–100 mg.	396	332	293	272	242
120–130 mg.	425	319	301	274	263
Mean growth rate (weeks 8–32):					
Alevin weight 90–100 mg.	11·9	10·5	8·4	6·8	6·3
120–130 mg.	13·6	11·1	8·9	7·7	6·9
Weight at end of week 32:					
Alevin weight 90–100 mg.	6960	4160	2195	1385	1130
120–130 mg.	10900	3460	2555	1680	1390

differences between the rates of individuals of similar position in different groups. There were greater differences in size between larger and smaller fry in the same group than between fry of similar position, but derived from alevins of different weight.

THE EFFECT OF HEREDITY ON THE EARLY GROWTH OF FRY

All the fry used in these experiments had the same male parent, and their female parents, trout A and B, were of the same age, stock and size. The fifty smaller alevins descended from female B had a weight range which included that of one group of fifty alevins descended from female A. Since these two groups were grown in similar environmental conditions, any differences between their growth histories may be due to genetical factors inherited through the female parents.

Table 8. *Comparison of the specific growth rates (as percentage weight per week) of fry with the same male parent but different female parents*

A, fry derived from alevins of weight 95 mg. descended from female A.
B, fry derived from alevins of weight 90–100 mg. descended from female B.

Position in size order ...	1		5		15		20	
	A	B	A	B	A	B	A	B
Specific growth rate during weeks:								
0– 3	26·0	24·8	23·3	20·8	20·9	17·4	16·5	15·2
3– 8	16·8	14·6	13·7	13·2	9·2	11·2	7·9	8·5
8–14	11·6	15·7	11·5	13·8	9·1	9·7	7·4	5·7
14–20	4·5	14·0	4·3	11·9	6·3	10·5	3·5	8·1
20–26	9·5	9·5	8·6	10·3	6·5	7·1	3·5	7·2
26–33	10·6	8·6	6·3	6·8	2·5	1·0	—	—
Weight of fry in mg.:								
In week 20	1435	2005	985	1290	710	956	530	798
In week 33	5320	6960	2550	4160	990	1385	—	—

The records for these two groups of fry show that their growth histories were not identical (Table 8). Those descended from female A had initially a higher specific growth rate than fish with the same position in the size order of the other group. During the third to eighth weeks, the two groups had approximately the same specific rates; in the eighth to twentieth weeks, the fry descended from female B maintained these rates while those of the other group decreased; after the twentieth week, the rates of growth of the fry descended from female B fell to the same level as those of the others. Twenty weeks after the beginning of feeding, the fry descended from female B were markedly larger than individuals in the same positions in the size order of the group descended from female A, and they continued to be larger during the next 3 months until the end of the experiments.

Within each group there was the same relationship between the size of an individual relative to the others and its specific growth rate, and the difference in size between large and small individuals in the same group was greater than that between individuals in the same positions in the size orders, but descended from different female parents. Thus, while the growth patterns of the two families were different, the factor which had the most marked influence on the specific growth rate of the individual fish was their position in the size order of their group.

DISCUSSION

Factors influencing the growth rates of animals may be of three types: genetical, physiological and environmental. Higgins (1929) and Davis (1934) showed that the average growth rate of *Salvelinus fontinalis* can be increased by selection of breeding stock for rapid growth, and they suggest that *Salmo trutta* and *S. irideus* are equally responsive to selection. The results of the present experiments showed that, even within the inbred stock of a hatchery, offspring of the same male but different female parents have somewhat different patterns of growth.

Minot (1890) pointed out that for most animals the specific growth rate is highest early in life and decreases, though with decreasing acceleration, as animals increase in age and size. This negative acceleration of specific growth rate depends on the age of trout fry, not their size, and may be partly a physiological effect of ageing of the tissues.

It is generally assumed that environmental factors are the most important of those concerned with the growth of fish. The principal physico-chemical factors are: temperature, amount and intensity of illumination, rate of water flow and concentration of gases and chemical substance in solution; the chief biotic factors are: quality and quantity of food and inter- and intraspecific relationships with other fish. Experimental work with salmonid fry has shown the importance of light (Tryon, 1942) and of rate of water flow (Washbourn, 1936). The effect of artificial foods on fry growth has been investigated in many hatcheries, and Coleman (1930) found that raw liver was the best food.

Gray (1928) and Wood (1932) showed the importance of temperature during the development of trout ova, and Gray (1926, 1928) also found a correlation between the embryonic specific growth rate of *S. fario*, the amount of yolk and the size of the embryo. Ova with less yolk grew into perfect fish of smaller size than normal. Dahl (1918–19), Richmond (1924), Willer, Quednau & Keller (1930) and Higgs (1942) all concluded that the size of the ova affects the growth rate of fry, larger ova giving rise to fry which grow faster. In the present experiments, alevin weight had little effect on specific growth rate. In one family, alevins of different sizes grew at the same average specific rates, so that the larger alevins became slightly larger fry than the others; in the other family, the smaller alevins had initially a higher specific growth rate and grew into fry of the same size as those derived from the larger alevins. However, the range of alevin weight used in these experiments was not so great as in those quoted above, and all the fish in the present experiments were closely related to each other. The average size of ova is probably characteristic of the female parent under natural conditions (Richmond, 1924) and may be associated with genetical factors which affect the growth rate of the fry.

The most important factor influencing the specific growth rates of individual fry proved to be the size of a fish relative to that of the others in the tank. Removal of larger fry resulted in increased specific growth rates of smaller ones, while addition of larger fry depressed the specific growth rates of smaller fry. This difference in growth rate, with the larger fish growing faster than smaller ones, was found in every experimental group. Since the fry were allowed to eat as much as they would, competition for food should not have been the factor producing this effect, and, indeed, the smaller fish appeared to feed as readily as larger ones. It seems possible that a 'social hierarchy', depending on size, soon developed within each group of trout fry, and that this influenced the specific growth rates of the individuals.

Observations on teleost fish with aggressive intraspecific behaviour have demonstrated that social hierarchies may exist in groups of fish, as they do in groups of other vertebrates such as hens (Schjelderup-Ebbe, 1922; Guhl & Allee, 1944). Noble and his collaborators (1938, 1939) have experimented with *Xiphophorus*, *Hemichromis*, *Danio* and other genera, while Braddock (1945) used *Platypoecilus maculatus*. When two individuals of these species meet, they 'challenge' each other and one may bite or 'nip' the other. After several encounters one becomes 'dominant' to the other and will nip it without being nipped in return. Where several fish are kept together, they soon arrange themselves in a 'nip order', which may be a straight-line order or more complicated. Dominant fish tend to be larger than their subordinates, and may have better access to food and mates. They lose less weight during periods of starvation. Hierarchies of *Platypoecilus* last only 2 days on the average, but those of *Xiphophorus* may last for 2 or 3 weeks. Fish which normally swim in schools are less aggressive and their hierarchies are probably more complex and less easily investigated.

No aggressive behaviour comparable with nipping was observed among trout fry, but soon after they began to feed they developed the habit of remaining in definite positions in each tank unless disturbed. When some range in size had been established, it was possible to recognize individuals by their resting positions. Moon (1936) records similar behaviour among salmon parr in aquaria. Young trout fry are sometimes found in shoals in streams, and there is a range in specific growth rate among fry in the same stream. This can be shown by calculating their size at the end of the first year, using the annuli on the scales. For Straffan (River Liffey), for example, Went & Frost (1942) record a minimum length of 3·8 cm. and a maximum length of 14·2 cm. (the average being 8·6 cm.) at the end of the first year's growth. This difference in growth rate might be the result of genetical factors, or of the selective action of a factor such as food supply, but it might also result from the existence of a size hierarchy in a stream, with correlation between the position of an individual and its specific growth rate. There is no evidence to indicate how the specific growth rate of a fish may be affected by its position in the hierarchy, either in streams or in tanks. In smaller, less crowded groups, however, an individual's specific growth rate depends not only on the number of fish which are larger than itself, but on the proportion of fish which are larger and smaller.

There is also an effect of crowding on specific growth rates, and those fry which are more crowded than the optimum density have a lower level of growth rate. Retardation of growth by overcrowding may be caused by: (1) competition for a limited food supply, (2) mutual mechanical disturbance causing increased activity and thus increased food requirements, (3) accumulation in the water of excreta and other metabolic products. This last factor, the 'conditioning of the medium', occurs especially in confined spaces such as aquaria and fry ponds, and may be beneficial to some extent (Allee *et al.* 1934, 1936, 1940). The first factor, competition for a limited food supply, should not have been operative in the present experiments, but the second or third factor might explain the retarded growth of the more crowded fish.

Pentelow (1944) suggested that the population density of trout is greater in soft than in hard waters, because of the superiority of spawning grounds and absence of predatory species of fish. Frost (1945), however, found no evidence in support of this suggestion for the River Liffey. The productivity of the limited volume of water in an aquarium is greatest for a medium population density, if the food supply is not limited, and increase in density leads to decrease in the level of specific growth rate. If the soft waters really are overcrowded with trout fry, this could be one factor contributing to the differences in specific growth rate between fry in the two types of water. Since, however, fry in hatcheries grow less rapidly in soft waters, there may be a direct effect of the chemical composition of the water on the growth of trout fry.

SUMMARY

1. Groups of trout fry of the same parentage were grown in environments where the following factors were controlled: temperature, amount and intensity of illumination, rate of water flow, aeration and chemical composition of the water, amount of living space and quality of food supply. They were allowed to eat as much as they would, and individual weights were recorded during the first 8 months after the beginning of feeding.

2. There was soon an increase in the range of individual weight in each group of fry, and thereafter the larger fry grew faster than smaller ones. When the larger fry were removed, the smaller ones grew at an increased specific rate, and when larger fry were added, the smaller ones grew more slowly. It is suggested that a 'size hierarchy' was established within each group, and an individual's specific growth rate depended on its position in the order of decreasing weight.

3. There was an optimum degree of crowding for maximum productivity. Compared with the fry in this group, the specific growth rates of individuals in larger, more crowded groups depended on the number of fish of larger size, while in smaller, less crowded groups, individuals grew at rates depending on the proportion of fish which were larger and smaller.

4. Alevin weight had little effect on the specific growth rates of fry.

5. There were differences between the growth histories of fry derived from alevins of the same weight and descended from the same father but different mothers (all of the same stock, age and size).

6. The specific growth rates decreased as the fry grew older, but there was no correlation between body weight and specific growth rate, except for the size hierarchy effect within each group. This effect had a greater influence on the size of individual fry than had either alevin weight or heredity.

This work was carried out while holding a Research Studentship and later an Assistant Lectureship at Girton College, Cambridge. I should like to thank Prof. J. Gray, F.R.S., for his advice and encouragement throughout.

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THE GROWTH OF BROWN TROUT (*SALMO TRUTTA* LINN.)

II. THE GROWTH OF TWO-YEAR-OLD TROUT AT A CONSTANT TEMPERATURE OF 11·5° C.

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(With Ten Text-figures)

Since fish form an important source of human food, a considerable amount of data is available concerning their distribution and size under natural conditions. This is especially true for the Salmonidae because of their popularity with sporting fishermen. With the development of commercial hatcheries, there has been a certain amount of experimental work concerning the effect of various factors on the growth and life history, particularly of 'trout'—*Salmo trutta*, *S. irideus* and *Salvelinus fontinalis*. However, it is impossible to define the effect of any particular factor on trout growth, since several environmental factors varied at the same time without being measured accurately. In some experiments (Surber, 1935; Pentelow, 1939) individual trout were isolated and their growth recorded, while in others (McCay & Tunison, 1935, 1937; Wingfield, 1940) the fish were kept in groups and their average sizes recorded at intervals. There is thus no record of the effect of intraspecific relationships on the growth of individuals.

The investigation now to be described was an attempt to study the growth of individual trout when the environmental factors were as completely controlled as possible. Observations on the early growth of trout fry under such conditions have already been reported (Brown, 1946), but these are very delicate and are difficult to identify as individuals in the early stages, so 2-year-old trout were used for a more detailed investigation, especially of the relation between food and growth in different environments. The present paper is an account of observations on trout living at a constant temperature of 11·5° C., and experiments concerned with the effect of temperature on growth, will be reported later.

The following factors were controlled or recorded throughout: temperature, amount and intensity of illumination, rate of water flow, chemical composition and aeration of the water, quality and quantity of food, volume of living space and number of individuals in each group. The following were investigated: the growth of individuals in groups, the effect of crowding on growth, changes in growth rate with age, the relation between food and growth and the effect of duration of light on growth. The number of individuals studied was limited by the space and time available, but the observations are published since some of the results were unexpected.

APPARATUS AND TECHNIQUE

The brown trout used in these experiments were obtained from the Midland Fishery, Nailsworth, Gloucestershire. They had been bred at the hatchery, and each consignment was all of the same age and of similar sizes, so that they had grown at the same average rates during the 18 months they had lived in the hatchery.

The fish were kept in tanks in a constant-temperature room which was set at 11·5° C., this temperature being chosen because it lies within the optimum range postulated by Pentelow (1939) and Wingfield (1940). The tanks were of glass, with angle-iron edges and slate floors, and they were arranged in two tiers, one at bench level and the other on the floor. The water supply came from a constant-level tank on the roof of the room, so that it was under constant pressure. On entering the room it flowed through a coil of metal tubing in the air and then through another coil immersed in standing water. This reduced its temperature to that of the room. The temperature was recorded by a thermograph in one of the tanks, and it seldom varied more than 0·2° from 11·5° C.

A T-junction distributed the water to each tier of tanks, and glass siphons between the tanks and an overflow device from the last one in each series maintained a constant level. The rate of flow was adjusted so that the water never became foul in any of the tanks, and it was then maintained constant. The siphons between the tanks were enclosed in cylinders of fine wire gauze so that particles of food should not be swept from one into the next, and certain tanks were divided by closely fitting glass plates into two spaces of known volume.

Cambridge tap water, an analysis of which was given by Brown (1946), was used throughout. All the tanks were supplied with compressed air, which bubbled through continuously and kept the water stirred. It was adjusted so that the water movement was not sufficiently violent to disturb the fish, while keeping the whole volume well aerated. The room was blacked out and the tanks were lit by 40 W. electric bulbs suspended 36 cm. above the water and arranged so that there was one for each 20 sq.cm. of water surface. The lamps were controlled by a clockwork time switch, and most of the tanks received 12 hr. of light and 12 hr. of darkness each day. One tank was surrounded with cardboard shields and a curtain of black material and its lamps were controlled by a second time switch, so that the fish in it could be allowed different amounts of light of the same intensity as that in the rest of the room.

The tanks did not all contain the same volume of water, and it was arranged that tanks of equal volumes contained different numbers of fish while tanks of different volumes contained equal numbers of fish, thus comparing the effects of the total amount of living space and of the amount of living space per individual on the growth of trout. Two of the tanks received water direct from the supply, while the rest received water siphoned over from another tank. The inhabitants of tanks of the same volume, but receiving fresh and conditioned water, were exchanged at intervals, so that any effect of 'conditioning' might be detected, though no effect was observed. All the tanks were covered with glass plates to prevent the fish from leaping out and the water from being contaminated with dust.

The fish were fed every day except Sundays and were given coarsely minced meat, from which the fat and gristle had been removed, mixed with about one-half its volume of minced liver. From a dish of this mixture, rations were weighed out for each tank and a sample was set aside for dry-weight determination. In order that the water should not become foul, the amount added to each tank was adjusted so that nearly all of it was eaten by the fish. Three times a week, as much as possible of the excess food and faeces in each tank was siphoned out, filtered with a Büchner funnel, and dried in an air oven at 80° C. for at least 20 hr. The dry weight of the excess food and faeces, subtracted from the dry weight of food added to the tank, gave the amount which had been absorbed by the fish. This amount is a maximum estimate, since some was probably lost by solution in the water and some may have been swept away through the gauze gratings. The food consumption has been expressed as milligrams of 'standard meat' per gram of fish per week, the percentage dry weight of the standard meat being 22·5.

The fish were generally weighed and measured once a fortnight, and they were starved on the previous day. Each was held with a damp cloth on a measuring board, and the length between the tip of the nose and the fork of the tail was read to the nearest millimetre. Each fish was then transferred to a waxed wooden box containing some damp cotton-wool. One box measured 20 × 5 × 5 cm. and another 30 × 5 × 5 cm., and each had a waxed wooden lid which could be held firmly in position with a rubber band. The box containing the fish was weighed to the nearest tenth of a gram, and the fish was then placed in water with ice floating in it. The box was weighed again and the weight of the fish was obtained by subtraction. The process of weighing and measuring occupied about 3 min. for each individual, and trout did not appear to suffer from the treatment if they were returned to cool, well-oxygenated water.

The individual trout differed among themselves in the shape of such parts as the operculum and in the number and arrangement of spots on the body. It was therefore possible to recognize individuals by their appearance and to keep individual records of their length and weight. Fish were kept for at least 6 weeks under the experimental conditions before observations on their growth were begun.

Most of the trout remained healthy, though a few suffered from disease. When a fish died, it was dissected to determine its sex and the state of its gonads. It was then either dried in the air oven at 80° C. in order to determine its dry weight, or was preserved in 4% formalin solution. Some individuals were examined thoroughly for parasites, from which they were remarkably free. Samples of scales were taken from all the dead fish, and have been preserved for future examination.

THE GROWTH RATES OF INDIVIDUAL TROUT

The individuals in each experimental group belonged to the same consignment from the hatchery and were of uniform size when the observations began. They had been living in the same conditions at the hatchery and were the same age, so that they had grown at the same average rates before the experiments began. Soon afterwards, however, the dispersion of individual weights within each group increased, the large fish becoming much larger than the smaller ones.

In Fig. 1 the fish in each group are arranged in order of decreasing weight, and their positions in this order are shown at intervals of 3 months. The largest fish generally remain at the top of these orders and the smallest ones at the bottom, but there are interchanges of position among the intermediate fish.

Specific growth rates have been calculated for each individual from the formula

$$G=\frac{\log_e Y_T-\log_e Y_t}{T-t}\times 100,$$

where G = specific growth rate, Y_T = weight or length at time T, Y_t = weight or length at time t, and T is later than t. The time was measured in weeks, so that the specific growth rate is expressed as percentage size per week.

In Fig. 2 individual specific growth rates have been plotted against individual positions in the size orders. Fish with high specific growth rates occur in positions 1–2, 4–5–6 and 9–10–11 in the orders of decreasing weight, while those in positions 3–4 and 7–8 grew at low rates. These maxima and minima are variable, but can be seen in the records for all groups of six or more fish. In these groups the fish at the head of the order is generally growing at a maximum rate and that at the bottom at a minimum rate, so that they remain in these positions. The fish in positions 5 and 10 approximately have high rates and should grow larger than those in positions 4 and 9. In the next 3-monthly period, however, the latter will have the higher specific rates and should regain their original positions in the order. Deviation from this pattern of specific growth-rate distribution among the members of a group was not uncommon.

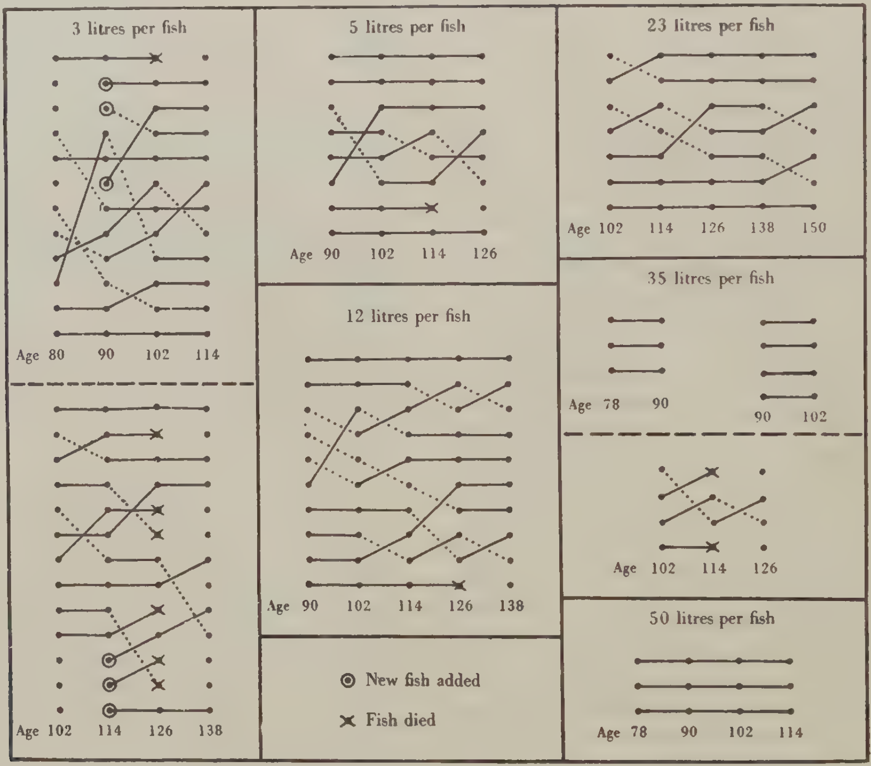

Fig. 1. Diagrams showing the positions of individual trout in the size orders of their tanks at 3-monthly intervals (their age is given in weeks). Full lines: fish maintaining their position or rising in the size order. Dotted lines: fish losing their position in the size order.

The effect of crowding on specific growth rates

The tank space in the constant-temperature room was divided up so that fish might be grown with the following amounts of living space per individual: 3, 5, 12, 23, 35 and 50 l. The number of fish which could be grown with the larger amounts of living space was necessarily small, and it was difficult to maintain these experiments because the trout frequently developed 'tail-rot' and died, so the conclusions about growth under these conditions are very tentative. The more crowded trout seemed to be much more resistant to disease, but had a high mortality on the few occasions when the compressed air supply failed, while the fish with intermediate amounts of living space survived. All the tanks received the same water supply, the same amount of aeration and illumination, and were at the same temperature. All were supplied with more food than the fish would eat. They thus differed only in amount of living space and number of individuals.

In all the experiments there were considerable differences between individual specific growth rates, and these were associated with individual position in the size orders. When the averages for each tank are compared over 3-monthly periods, there appears to be an optimum amount of living space per fish for rapid growth, and the average specific rates decrease for volumes greater and less than this amount (Fig. 3). The standard deviations from the means are large compared with the mean values and the numbers of

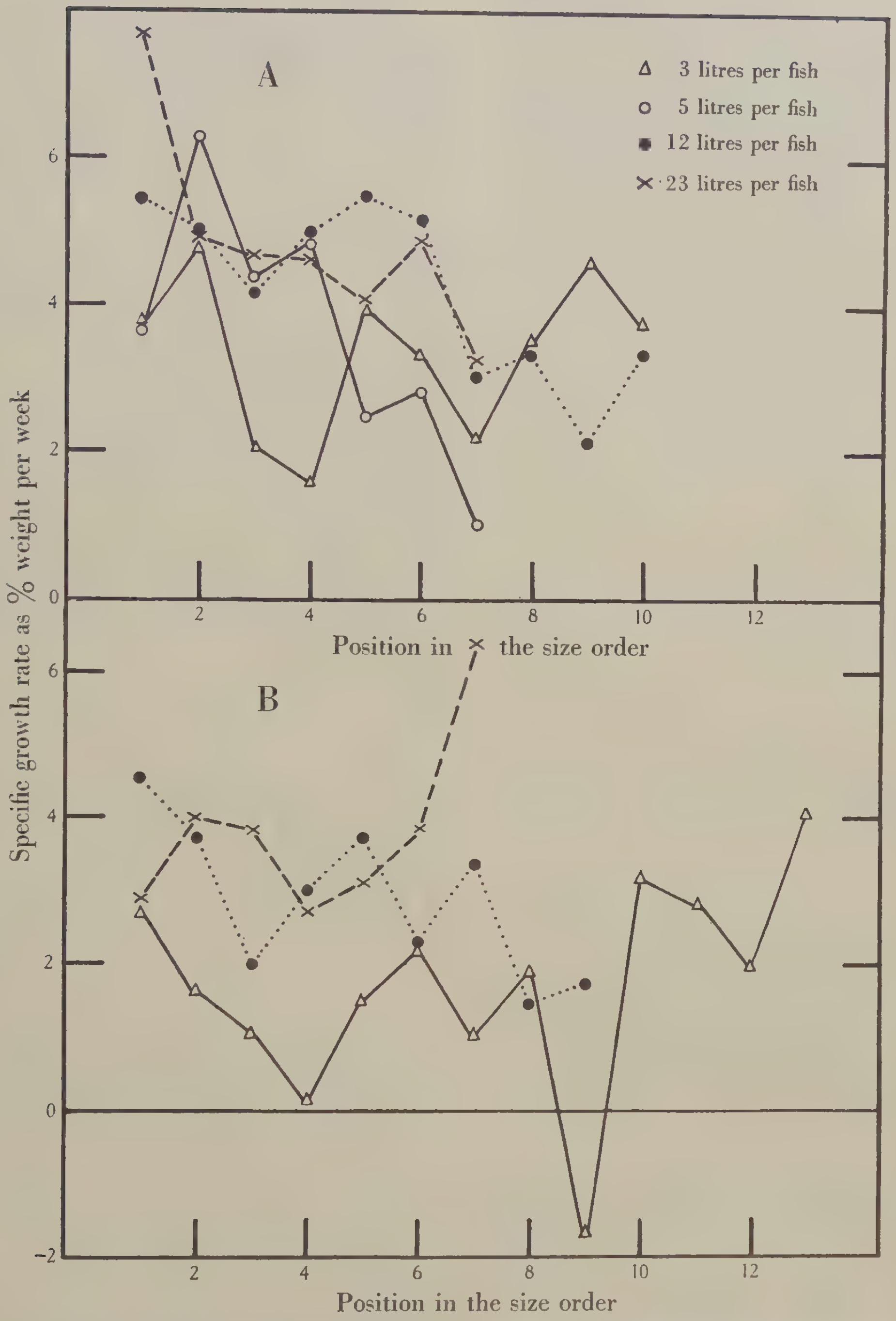

Fig. 2. Mean specific growth rates (as percentage weight per week) of individual trout with different amounts of living space per fish. A, fish aged 114–126 weeks. B, fish aged 126–138 weeks.

fish are not large, so the differences between the means are not statistically significant. Comparison of individual specific growth rates, however, shows that the values for fish in the most crowded tank were consistently lower than those for fish in the same position in the size order, but with more living space, while the few fish with large amounts of living space generally grew at lower rates than the corresponding more crowded fish (Fig. 2).

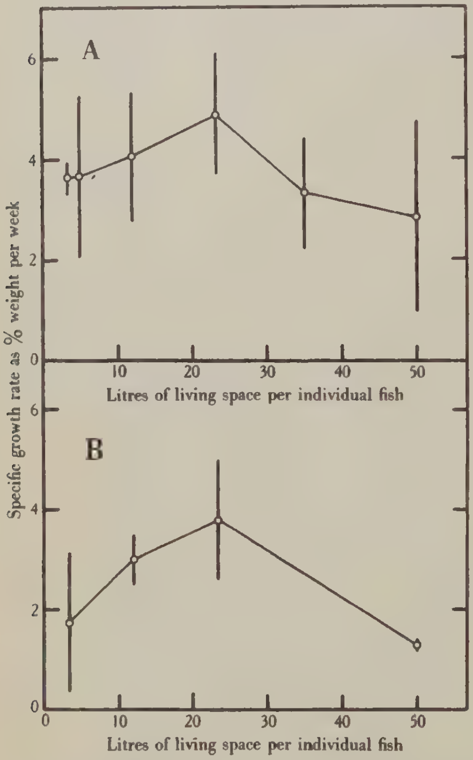

Fig. 3. Mean specific growth rates (as percentage weight per week) of groups of trout with different amounts of living space per fish. (The height of the vertical lines = 2 × the standard deviation from the mean.) A, fish aged 114–126 weeks. B, fish aged 126–138 weeks.

This effect of living space was not merely a correlation between the number of fish in a group and the average specific growth rate, since equal numbers of fish were kept with 5 and 23 l. per fish and approximately equal numbers with 3 and 12 l. each. There was also no obvious correlation between the total volumes of the tanks and the growth of the fish, since the two most crowded tanks each held 40 l., those with 12 and 35 l. per fish held 120 l., and the other two, with 23 and 50 l. per fish, held 160 and 150 l. respectively. Thus the effect depended on the degree of crowding (i.e. the amount of space per individual).

Annual changes in the specific growth rates

There were considerable differences between the specific growth rates of individuals in the same tank during each fortnight, but the average monthly specific rates of all the fish show an annual cycle of variation. For each tank, the standard deviation from the mean rate remained fairly constant throughout the year, and the growth rates of all the individuals varied in the same way.

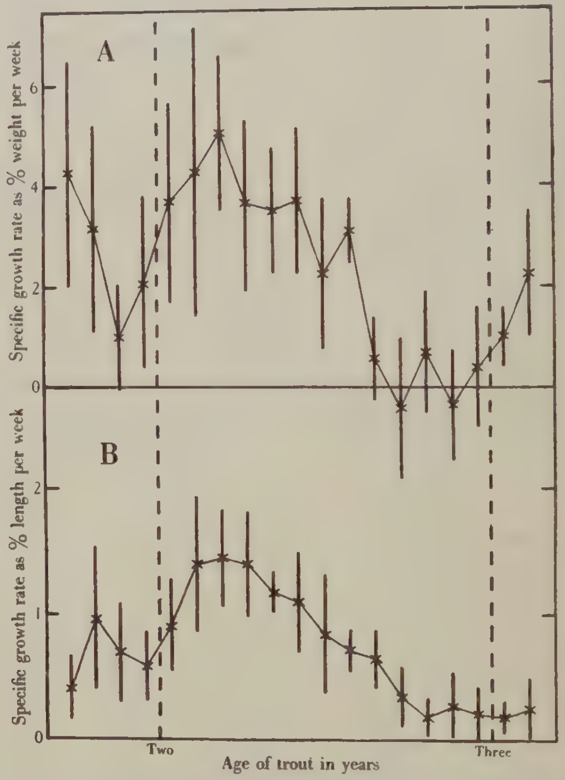

Fig. 4. Mean monthly specific growth rates of ten trout living in 120 l. of water and allowed to eat as much food as they would. (The year starts on 1 December and is divided into 13 months consisting of 4 weeks each. The height of the vertical lines = 2 × the standard deviation from the mean.) A, specific rate as percentage weight per week. B, specific rate as percentage length per week.

The mean specific growth rates calculated from the weight and length data for the ten fish with a living space of 12 l. per fish are shown in Fig. 4. For weight the specific growth rate fell to a low value in the autumn of 1942. The fish became 2 years old in December 1943, and the growth rate rose to a maximum in February 1943 and remained high till the late summer. It then fell markedly, and the behaviour of the fish changed at the same time. Instead of remaining inactive except when being fed, they became very restless and chased each other round the tank. In September, several of the larger fish died, apparently because of asphyxia, and on dissection they were found to be nearly sexually mature. The survivors were combined with the survivors in an adjacent tank. These were the same age and had had a similar growth history. Their growth rate continued to be low, and they were stripped of ova and milt in November 1943. They became 3 years old in December 1943, and the growth rate rose again in January 1944.

For length the specific growth rate followed the same cycle. There was a minimum in the autumn of 1942, a high rate from January to March 1943, and the rate decreased steadily to a minimum in the autumn of 1943 and rose again in January 1944.

This growth-rate cycle, with a low specific rate in the autumn, a maximum in early spring, a high summer rate and a decrease in the next autumn, accompanied by maturation of the gonads, occurred in all the groups of trout at 11·5° C., and the average rates for all the groups varied in the same way at the same times of year. During the whole of this period, however, there was no variation in any physico-chemical factor and the fish were allowed to eat as much as they would. This cycle must therefore reflect physiological changes in the individual trout. The onset of sexual maturity, which was accompanied by depression of the specific growth rate, occurred at the same time as it would have occurred had the fish been living in a hatchery. There was no correlation between the size of the fish and maturation of the gonads, since all became mature at the same time although they differed considerably in size.

Thus, 2-year-old trout, living in constant conditions of temperature, illumination, food supply, rate of water flow, and aeration and chemical composition of the water, had an annual growth-rate cycle, with slow growth in the autumn and rapid growth in early spring, decreasing during the summer to a 'check' in the autumn. They became sexually mature when they were just 3 years old.

Short-period changes in specific growth rate

In all the tanks at 11·5° C. most of the fish showed an alternation of periods of rapid and slow growth in weight and similar, though less well-marked, fluctuations in the rate of growth in length. For weight a period of rapid specific growth rate was followed by one of slow growth, and this was followed by another period of rapid growth, each period being about 2 weeks long. The closer the specific growth rate was to the average value (about 3–3·5 % weight per week), the less marked was the fluctuation. The specific rate of growth in length for a given period generally showed a direct correlation with the specific rate of growth in weight during the preceding fortnight. Thus, fluctuations in the rate of growth in length alternated with those in the rate of growth in weight.

The 'condition factor', K, has been calculated for each fish for each period from a formula generally used for brown trout:

$$K = \frac{100\,W}{L^3},$$

where W = weight in g., and L = length in cm. When the specific rate of growth in weight is correlated with this condition factor (Fig. 5), fish in very poor condition have a high specific growth rate; there is a growth rate minimum for condition about 0·93, and a maximum for condition about 1·07, and the growth rate is low above this value, so that fish in very good condition may lose weight. The specific rate of growth in length shows a direct correlation with the condition factor, good condition being associated with rapid and poor condition with slow growth.

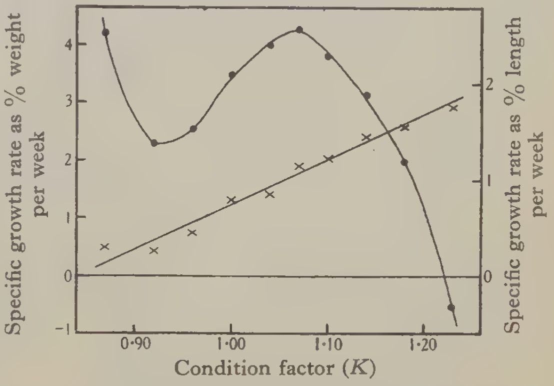

Fig. 5. To show the relation between the specific growth rates and the condition factor (K). ● specific rate as percentage weight per week. × specific rate as percentage length per week.

A picture of the growth of an individual trout may be obtained by combining these correlation curves. Table 1 shows growth-rate values which have been calculated for fish weighing 50 g. with different condition factors. The relation between the specific rates for growth in weight and in length is such that for fish with condition factors below about 1·08 the factor increases after a fortnight of growth, while there is a decrease in condition factor for fish with an original factor above 1·08. The greater the divergence of the condition factor from 1·08, the greater is the change in value towards this figure. The fluctuations in the specific rates of growth in weight and in length are thus more marked, the more the condition factor differs from 1·08.

THE RELATION BETWEEN FOOD AND GROWTH

Katabolic changes are always occurring in living cells, so that a certain amount of food must be utilized to replace the energy dissipated and the protoplasm broken down. Starved animals lose weight, and an animal must eat a definite amount of food, the 'maintenance requirement' or 'maintenance ration', to avoid loss of weight. Since it is necessary to know the maintenance requirement for a given set of environmental conditions before the efficiency of growth can be calculated, and since the maintenance requirement may change as an animal grows, experiments were designed to determine the relationship between it and the body weight of trout.

The relation between maintenance requirement and body weight

A group of ten fish were kept in an 'unconditioned' tank in the constant-temperature room, and were weighed once a week. The change in their average weight was expressed as a proportion of their original weight (gain or loss as milligrams per gram of fish). The weight of meat absorbed was also measured and expressed as milligrams per gram of fish, and the two quantities were plotted against each other. At the 'maintenance level' there was a direct relationship between the amount of food absorbed and the change in weight, so the total amount of food added to the tank was reduced until at least three consecutive values showed this relationship. The value for amount of food absorbed for which no change in weight occurred was then taken as the maintenance requirement.

Table 1. *Comparison of the growth of trout weighing 50 g. with different condition factors during 2 weeks in the standard environmental conditions*

(Calculated from the data shown in Fig. 5.)

Condition factor (*K*) ...	0·88	0·94	1·02	1·07	1·14	1·20
Original weight (g.)	50·0	50·0	50·0	50·0	50·0	50·0
Original length (cm.)	17·7	17·5	17·0	16·7	16·4	16·0
Specific growth rate:						
As % weight per week	4·2	2·4	3·7	4·2	3·0	0
As % length per week	0·25	0·30	0·80	1·10	1·50	1·70
Final weight (g.)	54·4	52·4	53·8	54·4	53·1	50·0
Final length (cm.)	17·8	17·6	17·3	17·1	16·9	16·6
Final condition factor (*K*)	0·97	0·98	1·04	1·08	1·10	1·10
Change in *K*	+	+	+	+	−	−

As the rations were reduced towards the maintenance level, there was an increase in the efficiency of growth, but when the maintenance level had been reached, there was no further change in efficiency. Only the last three or four in each series of values have therefore been used to calculate the requirement. In one experiment, with fish of average weight 111·7 g., rations were kept at the maintenance level for eight consecutive weeks, and there was no change in efficiency. Fig. 10 A is a typical series of values giving a maintenance requirement.

After determination of a maintenance requirement, the fish were fed at a higher level until the average weight had increased to a new value. The amount of food was then reduced until another maintenance requirement could be calculated. At the maintenance level, all the fish in the tank gained or lost weight simultaneously, so it is assumed that each received a fair share of the food. During the intervals with higher rations some grew faster than the others, but all increased in weight.

The maintenance requirement decreased, relatively, as the fish increased in weight, reaching a constant level for about 100 g. average weight (Fig. 6). An unexpectedly low value was obtained in November to December 1943, with fish which were just 3 years old and must have been nearly sexually mature. After 1 month of increased food, two of them were stripped of ova (about 15 % of the body weight) and three of milt (about 5 % of the body weight). During two subsequent maintenance periods their requirements were at the higher level which had been expected from the earlier values. It seems likely that these fish had well-developed gonads. which had

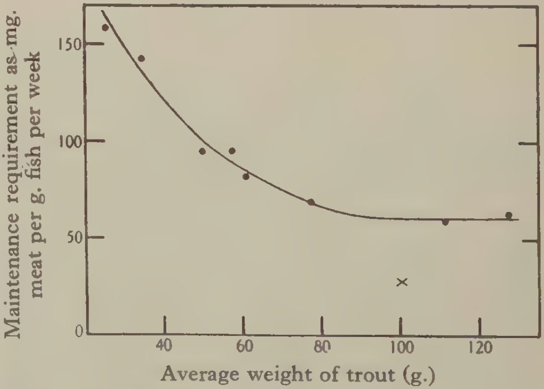

Fig. 6. To show the relation between maintenance requirement and body weight for trout at 11·5° C. ● fish with small gonads. × fish with well-developed gonads.

grown in size during the preceding growth period, and these were partially resorbed when the food rations were reduced.

The amount of food eaten and efficiency of utilization of trout with an unrestricted food supply

The total amount of food absorbed by the fish in each tank was measured, and the values have been divided by the total weight of fish in the tank so that they are expressed as milligrams of meat per gram of fish per week. The amount absorbed by ten fish in 120 l. of water is shown in Fig. 7. The amount varied from week to week but decreased as the fish grew older and larger, and was lowest, on the average, when the fish were aged 150 to 160 weeks, and had fully developed gonads.

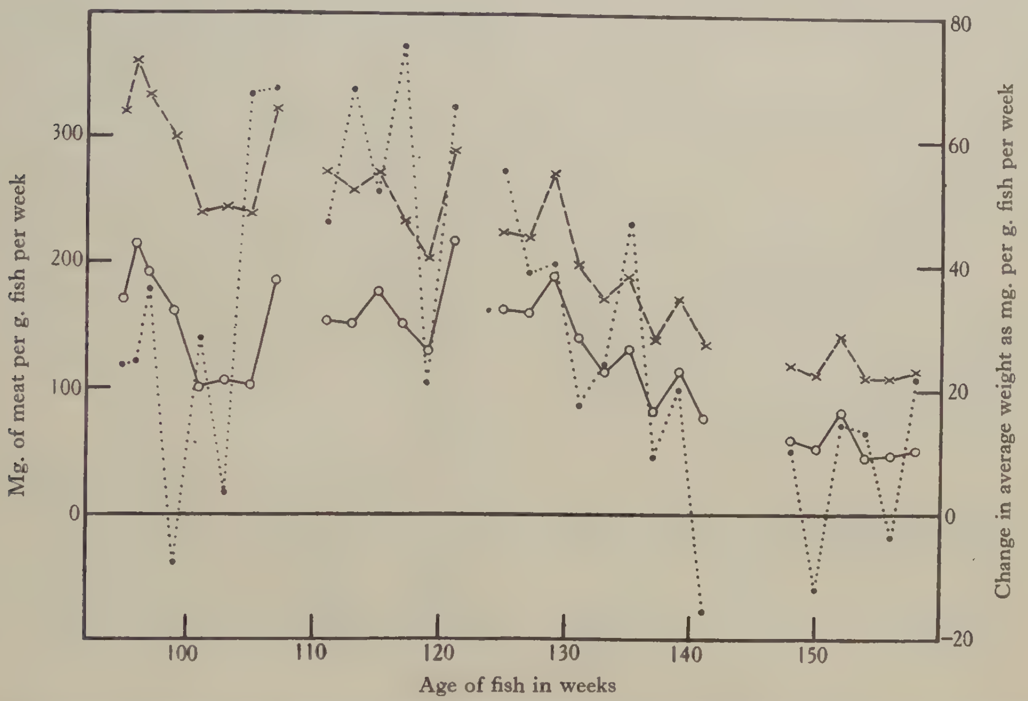

Fig. 7. To show the change with age in the feeding and growth of trout allowed to eat as much food as they would. × total amount of meat absorbed per g. fish per week. ○ amount eaten in excess of the maintenance requirement (per g. fish per week). ● change in average weight per g. fish per week.

Maintenance requirements decreased, relatively, as the fish grew larger, and the total amount of food absorbed must have been used for maintenance as well as for growth. The amount available for growth may be found by subtracting the maintenance requirement for fish of that average weight from the total amount absorbed during each week. This quantity varied from week to week (Fig. 7), but fluctuated round a constant level for fish aged between 95 and 140 weeks. There was no apparent correlation between it and the average weight of the fish.

The amount of food eaten in excess of the maintenance requirements has been correlated with the condition factor of the average fish. The relation (Fig. 8) is similar to that between the specific rate of growth in weight and the condition factor, but with the maximum at 1·12 instead of 1·08. Unfortunately, there are no values for fish with an average condition factor less than 0·95.

The change in average weight (expressed as milligrams per gram of fish per week) was roughly proportional to the amount of food eaten in excess of the maintenance requirements (Fig. 7), but that this relationship is not direct can be shown by calculating the 'efficiency of utilization' of the food. This is defined here as the increase in average weight (as milligrams per gram fish per week) per unit amount of food eaten in excess of the maintenance requirement (also as milligrams per gram fish per week). It may also be called 'efficiency of conversion of food into fish' or 'efficiency of growth', and it was not constant from week to week.

There is a strong positive correlation ($r = 0{\cdot}87$) between the efficiency and the amount of increase in average weight (Table 2). Larger increases in weight were more efficient than smaller increases, so that the former required relatively less food. Since rapid growth in weight was associated with high efficiency, the curve correlating efficiency with the condition factor resembles that correlating the specific rate of growth in weight with the condition factor. There is a maximum at about $K=1{\cdot}12$ (Fig. 8), and again there are no values for fish with an average condition factor less than 0·95.

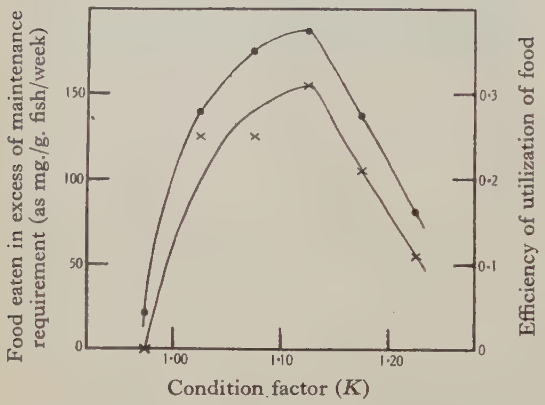

Fig. 8. To show the relation between the amount of food eaten in excess of the maintenance requirement, the efficiency of utilization and the condition factor (K). ● amount of food eaten in excess of the maintenance requirements (as mg. of meat per g. fish per week). × efficiency of utilization of the food.

Table 2. *The relation between increase in weight and efficiency of utilization of food for fish with unrestricted and restricted rations*

Increase in weight mg. per g. fish per week	Efficiency of utilization	
	Unrestricted rations	Restricted rations
0–10	0·08	0·07
10–20	0·16	0·13
20–30	0·22	0·31
30–40	0·21	0·75
40–50	0·30	0·60
50–60	0·37	—
Over 60	0·44	0·60

These observations are based on the feeding and growth of the average fish, and they support the conclusions derived from growth records for individual trout, that short-period fluctuations in the rates of growth in weight and in length are related to the condition factor. The specific rate of growth in length varies directly with this factor. The amount of food eaten in excess of the maintenance requirements and the efficiency with which this food is used for growth are both high for a condition factor of about 1·10, and growth in weight is most rapid for fish with approximately this condition factor. With higher and lower condition factors, the amount of food eaten and the efficiency are lower, and so is the specific rate of growth in weight. Unfortunately, there are no figures for the food eaten and efficiency of fish with an average condition factor less than 0·95, but, by analogy with the specific rate of growth in weight, they should be high if the condition factor is less than 0·90.

The effect of crowding on the amount of food eaten and efficiency of utilization of the food

Assuming that maintenance requirements vary with the weight of the fish and are independent of the amount of living space, the food eaten in excess of these requirements has been calculated for each tank in the constant-temperature room (Table 3). For each group of fish, the values were independent of the average weights and fluctuated about a mean. There were no marked differences in the amount of food eaten by fish with amounts of living space between 5 and 23 l. per fish; those with 3 l. per fish ate less food and showed less variation in the amounts eaten from week to week; while those with 50 l. per fish showed marked fluctuations in the amount eaten, which in some weeks was little more than their maintenance requirements. The average efficiency of utilization of the food was low in the more crowded tanks compared with fish with greater amounts of living space (Table 3).

The degree of crowding thus has little, if any, effect on the feeding of fish with moderate amounts of living space. Very crowded fish show less variation in the amount of food eaten, eat less food and use it less efficiently, while those with large amounts of living space show large fluctuations in the amount eaten and in efficiency. These observations on feeding thus support the conclusion that over- and undercrowding both lead to reduction in the average specific growth rate, so that there is an optimum population density for rapid growth.

The growth of trout with a restricted food supply

In order to investigate more thoroughly the relation between the amount of food eaten and the change in body weight, seven fish in 40 l. of water were given amounts of food which were more than their maintenance requirements but less than the amounts which they would have eaten if not restricted. It was intended that their rations should be kept at a constant level above the maintenance requirement, but fluctuations in the dry weight of the food made this impossible. The fish were weighed and measured once a week, and they all survived until the end of the experiment. They all gained and lost weight in the same way, so it is assumed that they each obtained a fair share of the food. Their average condition factor varied between 0·95 and 1·00.

Fig. 9 shows the amount of food that was added to the tank and the amount that was actually absorbed by the fish (both expressed as milligrams per gram fish per week). The latter quantity generally varied directly as the amount added, but in some weeks, e.g. when aged 115, 122, 127–130, 136 and 139 weeks, the fish did not take full advantage of their food supply.

Table 3. *The amount of food eaten in excess of the maintenance requirements and the efficiency of utilization of food of trout with different amounts of living space per individual*

Litres of living space per fish ...	3	5	12	23	35	50
Mean amount of food eaten in excess of maintenance requirement (mg. meat per g. fish per week)	133 ±9	174 ±50	163 ±35	176 ±50	148 ±29	141 ±78
Mean efficiency of utilization	0·08	0·17	0·22	0·23	0·19	0·26

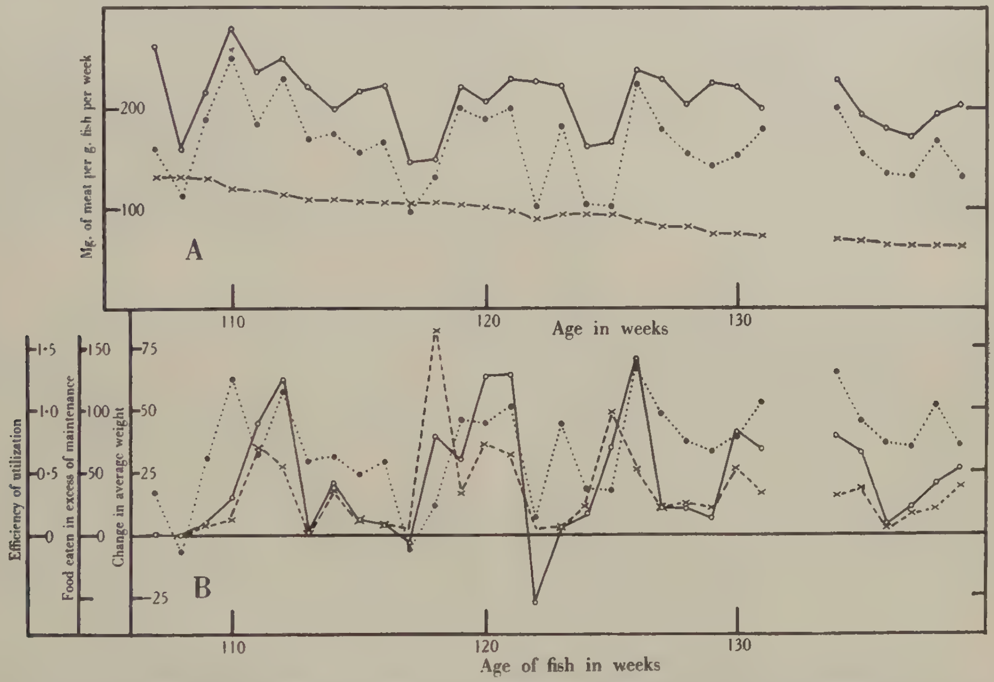

Fig. 9. To show the feeding and growth of trout with a restricted amount of food. A: ○ amount of meat added to the tank per g. fish per week; ● amount of meat absorbed per g. fish per week; × maintenance requirement (as mg. meat per g. fish per week). B: ○ change in average weight per g. fish per week; ● amount of food eaten in excess of the maintenance requirement (as mg. meat per g. fish per week); × efficiency of utilization of the food.

When the maintenance requirements are subtracted from the amount of food eaten, to give the amount available for growth, this quantity shows considerable variation from week to week, but it was never as high as the average amount available to fish with unrestricted rations.

The weekly change in average weight is also shown in Fig. 9; it was very variable, a period of rapid increase being followed by a period in which there was no gain and sometimes even loss of weight. The peaks in the growth curve generally coincided with peaks in the curve for food eaten in excess of the maintenance requirement, while weeks in which little food was eaten generally followed periods of rapid increase in weight. The efficiency of utilization of the food (Fig. 9) was generally at a maximum value after a period of poor growth and low food intake, and these maxima preceded maxima for change in weight and food eaten in excess of the maintenance requirements.

Table 2 shows that, for changes in weight greater than 20 mg. per g. fish per week, the efficiency of growth was markedly higher than that of fish of the same age with unrestricted rations.

Thus 2-year-old trout with restricted rations showed cycles of growth and rest. A period of little growth in weight and small amount of food eaten was followed by one of increased food intake associated with high efficiency. The latter fell as the food intake increased, and the change in weight rose to a maximum with the amount of food eaten. The peak of rapid growth, which lasted for 1 or 2 weeks, was followed by decrease in food intake and consequent poor growth. The cycle was then repeated, taking between 6 and 8 weeks. Growth in length occurred alternately with growth in weight, as with an unrestricted food supply.

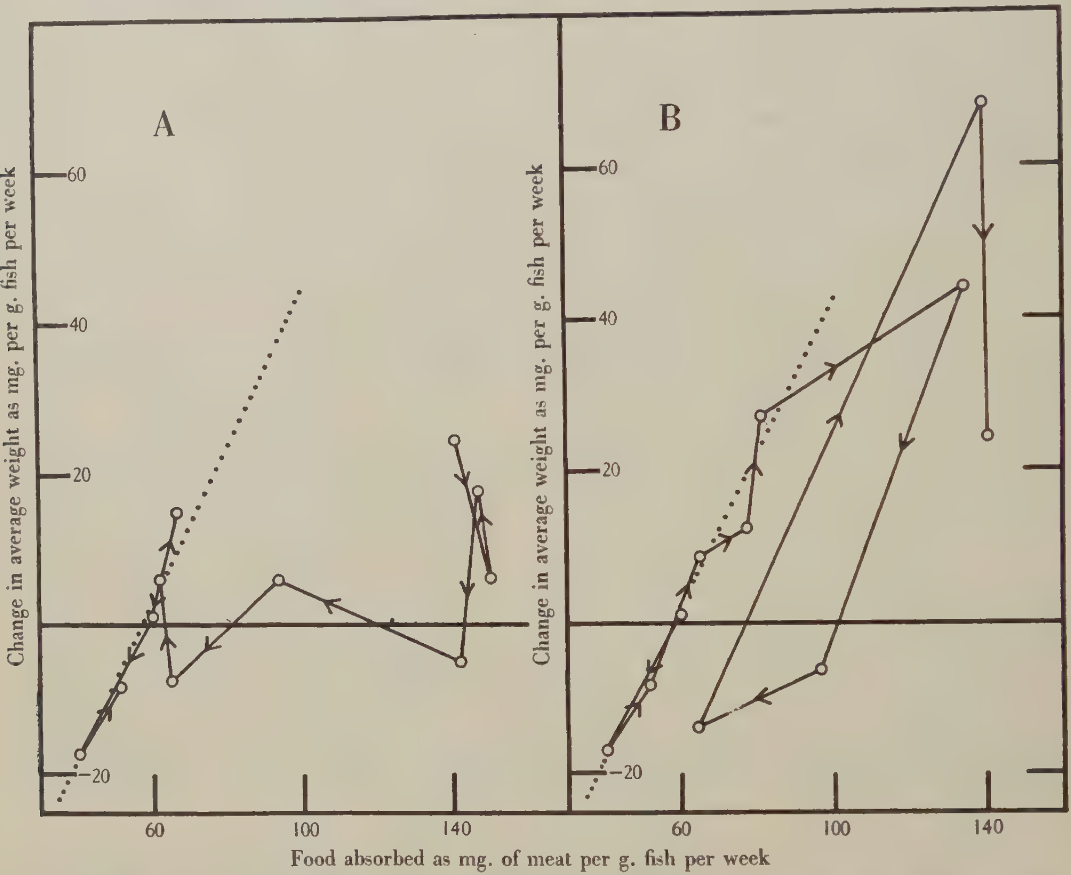

Fig. 10. Adaptation of trout to different levels of feeding. A: the amount of food added was reduced towards the maintenance level. B: the amount of food added was increased above the maintenance level. The dotted line represents the relation between the amount of food absorbed and the change in average weight at the maintenance level. Values for consecutive weeks are joined by straight lines, and the arrows show the order in which the values were obtained.

When growth in weight occurred, the efficiency was greater, so that the fish with a restricted food supply attained a size which was not very much smaller than that of fish of the same age with unrestricted rations. The former grew spasmodically, possibly by exaggeration of the short-period fluctuations in growth rate, but their rapid growth periods compensated for the intervening periods of little or no growth.

The effect of altering the level of feeding on the growth of trout

As the amount of food added to the tank was reduced during each determination of a maintenance requirement, the fish first lost weight, then became 'adapted' to the smaller amount of food and gained weight. If the amount of food was reduced further, they again lost weight for a week, then became adapted once more and gained weight. When the maintenance level was reached, however, the change in weight was directly proportional to the amount of food absorbed, and this relationship remained constant.

This 'adaptation' to reduced levels of feeding was observed during every determination of maintenance requirements, and a typical record is given in Fig. 10A.

Fig. 10 B shows the effect of increasing the rations of fish which had lived for 6 weeks at the maintenance level. At first they were very efficient, but they soon became adapted to the higher level of feeding, and a slight reduction in the amount of food then led to loss of weight, although the amount eaten was greater than the maintenance requirement. After another week at this lower level, however, they became adapted to it, and an increase in the amount of food again resulted in rapid increase in weight. During the following week, with the same amount of food, there was less than half as much increase in weight, the efficiency having again decreased.

Trout therefore use their food more efficiently when the amount is restricted, and they become adapted to different levels of feeding in 1 or 2 weeks. At the maintenance level, growth in weight is directly proportional to the amount of food absorbed, but at higher levels there are cycles of alternating rapid and slow growth.

Table 4. *The specific growth rates of trout with different amounts of light*

Amount of light per day ...	6 hr.	12 hr.	18 hr.
Mean rates (as % weight per week):			
Fish aged 154–174 weeks	—	1·19 ± 1·07	1·14 ± 0·94
Fish aged 194–201 weeks	2·40 ± 0·71	0·51 ± 0·89	—
Mean rates expressed as % of those of fish of the same age with 12 hr. per day	473 ± 140	100 ± 120	100 ± 84

THE EFFECT OF LIGHT ON THE GROWTH OF TROUT

One 120 l. tank in the constant-temperature room was enclosed with cardboard screens and a black curtain so that it was shielded from the lights in the rest of the room. The two electric bulbs above the tank were connected to a separate time switch, and the six trout in the tank were grown for 5 months with 18 hr. of light per diem; the amount of light was reduced at the rate of 1 hr. per diem per week, and they were then grown for 2 months with 6 hr. of light per diem. They were allowed more food than they would eat, so that their growth should be directly comparable with that of fish of the same age in the other tanks.

The mean specific growth rates, calculated from the weight data, for fish of the same age with 6 and 12 and with 12 and 18 hr. of light per diem, are given in Table 4. There was no difference between the specific growth rates of fish with 12 and 18 hr. per diem, except that there was slightly less individual variation with the greater amount. Fish with only 6 hr. of light per diem, however, grew significantly better than those of the same age with 12 hr. ($t=3\cdot 1$, $P=0\cdot 01$).

These rather limited observations suggest that when trout are given as much food as they will eat once a day, growth is more rapid when the total duration of illumination is less than 12 hr. per diem, but increase in the duration above this amount does not result in further depression of the growth rate. No experiments were performed with lights of different intensities.

DISCUSSION

In the first paper of this series (Brown, 1946) it was shown that the factor which had the greatest influence on the early growth of individual trout fry in aquaria was the size of a fish relative to that of the others in the same group. The larger individuals grew faster, and if they were removed the smaller individuals showed increase in their specific growth rates. Among groups of 2-year-old trout, also, the relative size of an individual had an effect on its specific rate of growth. In groups of fry, an individual's growth rate was proportional to its position in the hierarchy of decreasing weight, but for the older fish the position was more complex. The largest and smallest individuals in each group generally retained these positions in the size order throughout the experiments, but there were exchanges in position among the intermediate fish, occurring about every 3 months. The individuals appeared to form 'subgroups' of three, four or five fish of similar weights, and within each subgroup the largest fish grew fastest and the smallest most slowly. After about 3 months the largest individual of one subgroup would be larger than the smaller ones of the next larger subgroup, and there was a rearrangement of hierarchies. There were, however, many exceptions to this pattern of growth-rate distribution.

The older fish, like the fry, were allowed to eat as much as they would, and the smaller individuals appeared to eat as readily as the larger ones. Thus, competition for a limited food supply cannot be invoked to explain the size hierarchy effect. The older trout, like the fry, soon developed the habit of resting in definite positions in their tanks, and if new fish were added to the tank and came to rest in one of these positions, the 'owner' would chase it away. Certain individuals were more aggressive and would chase the others round their tanks. Thus, within each group there were individual differences in behaviour, and at least some fish appeared to react to others as towards recognizable individuals. Every trout seemed to acquire certain territorial rights, and it is not inconceivable that individuals of approximately the same size formed subgroups within the larger group. Dominance-sub-ordination relations within the subgroups may then have determined the growth rates of the individuals. Such an effect of social dominance on growth rate was reported by Noble (1939), who also found that stable hierarchies could last for 2 or 3 weeks among *Xiphophorus*, but in these cases all the fish in the tank were involved in a single group.

Anglers often find that certain stations in rivers and lakes are occupied by large trout. If one of these is caught its position is taken, sometimes within an hour, by another fish, generally slightly smaller. There is thus evidence that there is a 'size hierarchy' in natural conditions, and that larger trout occupy more favourable parts of rivers and lakes.

The degree of crowding affects the level of growth rate within the group. Overcrowded fish eat less, use the food less efficiently and grow more slowly than fish with more living space. Those with a large amount of individual living space, however, are very susceptible to disease and grow and feed erratically and not so well, on the average, as those with intermediate amounts. The fish were exchanged at intervals between tanks with 'conditioned' and 'unconditioned' water, and there appeared to be no correlation between the type of water and the growth rate. The existence of an optimum degree of crowding for rapid growth therefore cannot be explained by the theory of chemical conditioning of the medium put forward by Allee (1939). There is also no evidence that the actual volume of the tanks limited the growth of the fish, since some of the larger fish grew in the smaller tanks, and vice versa.

Welty (1934) demonstrated that individuals of the genera *Notropis*, *Macropodus*, *Brachydanio* and *Carassius* ate more food when grouped than when isolated, the stimulus for extra feeding being the sight of other individuals feeding. Among the 2-year-old trout, those with the greatest amount of living space always rested far apart in the tank, and there was no evidence of mutual relations. The most crowded fish were continually disturbing each other and had a generally lower level of appetite and efficiency. Guhl & Allee (1944) found that a flock of hens in which there was continuous upheaval of the social structure ate less and did not grow so well as one in which there was a stable hierarchy, and it is possible that the subgroups were less stable in the more crowded tanks of trout than in those with intermediate amounts of living space per fish. In the latter, appetite and efficiency were high; there was probably little mechanical disturbance; there may have been mutual visual stimulation; and the hierarchies were probably stable.

As trout fry grow older, their specific growth rates decrease (Brown, 1946). The average growth rates of the 2-year-old trout were markedly lower than those of the fry, in accordance with the principle propounded by Minot (1890) that the acceleration of specific growth rate is negative. It might be expected that the decrease in rate would be gradual and continuous under constant environmental conditions. All the trout at 11·5° C., however, had an annual cycle of growth with an autumn check when they became 2 years old, rapid growth in the next spring, slower summer growth and another autumn check which coincided with maturation of the gonads as they became 3 years old. This cycle was presumably the result of an annual physiological cycle of changes in the internal environment, possibly the variation of secretion of an endocrine organ such as the pituitary gland. Hasler, Meyer & Field (1939) showed that extract of carp pituitary can induce premature spawning in rainbow trout, and pituitary extracts affect growth and reproduction in other vertebrates.

Since this cycle occurred in the absence of variation of any environmental factor it cannot depend on the existence of environmental 'time-markers'. These have been shown to be important in the maturation of the gonads of *Salvelinus fontinalis* (Hoover & Hubbard, 1937) and *Phoxinus laevis* (Bullough, 1939, 1940). The brown trout became sexually mature at the same age and at the same season as those of the same stock under natural conditions. Maturation of the gonads is generally followed by decrease in the growth rate (Fage & Veillet, 1938) and is therefore an important event in the growth history of fish. There was no apparent difference between the growth rates of 2-year-old male and female trout.

Within this annual cycle of growth, individuals showed short-period fluctuations in specific rate taking between 4 and 6 weeks and correlated with the condition factor. Trout in 'good' condition are heavier relative to their length than those in 'poor' condition, and probably contain more food reserves. Growth in length, which involves regional differentiation, occurs at a rate directly proportional to the condition factor and thus to the amount of reserve food. Fish in very good condition eat little food, while those in very poor condition probably eat plenty, i.e. appetite is low for fish with food reserves and high for those with none. The amount of food eaten is also high for trout in medium condition and low for those in poor condition. The efficiency of utilization of the food and the specific rate of growth in weight vary with the condition factor in the same way as the amount of food eaten. The balance between condition factor, growth in weight and growth in length is such that there are alternating periods of slow and rapid growth; rapid growth in length alternates with rapid growth in weight, and the condition factor tends towards a value of approximately 1·08.

When the level of feeding is reduced and the fish are allowed amounts between the maintenance requirement and the amount they would eat with unrestricted rations, the short-period fluctuations in specific rate of growth are exaggerated. Since the efficiency of utilization of the food is higher, on the average, these fish reach a size not very much smaller than that of trout of the same age with unrestricted rations. These observations agree with those of McCay & Tunison (1935, 1937) on *Salvelinus fontinalis*, for which the efficiency was inversely proportional to the level of feeding. When this level was altered, the brown trout became 'adapted' to the new level in 1 or 2 weeks. This was observed also by McCay, Dilley & Crowell (1929) using *Salvelinus* and by Moore (1941) using *Apomotis*, *Helioperca* and *Perca flavescens*.

At the maintenance level, the relation between the amount of food absorbed and the change in weight remained constant. The maintenance requirement decreased relatively with increase in weight of the fish, reaching a constant level at about 100 g. body weight. Pentelow (1939) estimated the maintenance requirements and efficiency of utilization of trout of average weight at about 20 g., but since most of his determinations were at temperatures between 45 and 50° F., his values are not comparable with those given in this paper. He found no correlation between maintenance requirements and body weight.

Surber (1935) estimated that individual *Salmo gairdnerii* of average weight about 50 g. required 6·63 g. of *Gammarus* at 12° C. for an increase in weight of 1·0 g. This amount of food included the maintenance requirement, which was not estimated, and the value calculated for *Salmo trutta* under the same conditions is 6·3 g. of meat for an increase in weight of 1·0 g., if allowance is made for the rate of growth. Dawes (1930) investigated the maintenance and growth requirements of plaice fed with *Mytilus*, and his values are of the same order as those for *Salmo trutta*. He found also that the plaice were more efficient at lower levels of feeding.

Wingfield (1940) remarked that in temperate zones the annual variation in temperature is accompanied by annual variation in the amount and intensity of light, but there has been little attempt to separate the effect of these two factors on growth. The rather limited experimental evidence reported in this paper showed that the growth of 2-year-old trout was more rapid with less than 12 hr. of the standard illumination per diem. No experiments were performed with different intensities of light, and it is possible that there is an optimum amount (duration × intensity) for growth. Light might affect the growth rate through the pituitary gland, which is known to mediate melanophore responses in salmonid fry (Neill, 1940).

Under natural conditions the duration of light may affect growth directly, as in these experiments, but trout feed by sight, so it will also affect the amount of time during which the fish may capture food. If the food supply requires much search, the duration of light might be a factor reducing the growth rate by limiting the amount of food which could be eaten each day. Theoretically, therefore, there should be an optimum length of day, depending on the 'availability' of food organisms, such that the duration of light is sufficiently long for feeding but short enough for growth not to be inhibited.

SUMMARY

1. Two-year-old trout were grown in environments where the following factors were controlled: temperature, amount and intensity of illumination, rate of flow, composition and aeration of the water, quality and quantity of food and amount of living space.

2. The specific growth rate of an individual depended on its size relative to that of the others in the group. It is suggested that subgroups of four or five individuals existed within the size hierarchy and were reorganized at intervals of about 3 months.

3. There was an optimum degree of crowding for rapid growth, and overcrowding led to lower appetite and efficiency of utilization of food, while under-crowded trout ate and grew erratically.

4. In spite of constant environmental conditions, all the fish had an annual growth-rate cycle, with an autumn check, a spring maximum, rapid summer growth and another autumn check, which coincided with maturation of the gonads when they became 3 years old.

5. Individual specific growth rates fluctuated over periods of 4–6 weeks, and rapid growth in length alternated with rapid growth in weight. The specific rate of growth in length was directly proportional to the condition factor. The amount of food eaten, the efficiency of utilization of food and the specific rate of growth in weight varied with the condition factor and were maximal for a factor of about 1·10.

6. The growth-rate fluctuations were exaggerated and the efficiency was greater when the food supply was restricted. At the maintenance level the change in weight was directly proportional to the amount of food eaten. The maintenance requirement decreased, relatively, with increase in body weight.

7. The mean specific growth rate was higher with less than 12 hr. per diem of the standard illumination.

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THE GROWTH OF BROWN TROUT (*SALMO TRUTTA* LINN.)

III. THE EFFECT OF TEMPERATURE ON THE GROWTH OF TWO-YEAR-OLD TROUT

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(*Received* 1 *October* 1945)

(With Six Text-figures)

Temperature is a limiting factor in the distribution of many animals. The ova of brown trout (*Salmo trutta*) can develop without excessive mortality at water temperatures between 3 and 15° C. (Gray, 1928), while for adult trout, mortality is high above 20 and complete above 25° C. (Gardner & Leetham, 1914; Audigé, 1921; Gardner, 1926). It is generally assumed that temperature is the most important physico-chemical factor affecting the growth of fish, especially in temperate zones, where there is an annual variation. Allen (1940) deduced from examination of salmon scales from British rivers that parr do not grow when the water temperature is less than 7° C. Pentelow (1939) and Wingfield (1940) observed the growth of yearling trout in tanks where the temperature varied with that of the external environment and was recorded once a day. They found that there was no growth at temperatures less than 6° C., and it was most rapid between 10 and 15° C. Since other environmental factors varied at the same time, the correlation between growth rate and temperature was necessarily approximate.

The present paper describes part of an investigation of the growth of trout when environmental factors were controlled as completely as possible. Observations on the early growth of trout fry and of that of two-year-old trout at a constant temperature of 11·5° C. have already been reported (Brown, 1946*a*, *b*). In the present experiments, all the environmental factors except temperature were similar to those in the constant-temperature room at 11·5° C.

APPARATUS

Two-year-old trout from the Midland Fishery, Nailsworth, Gloucestershire, were kept at different constant temperatures by using 120 l. tanks enclosed in insulating cases. Each of these consisted of two shells of wood substitute separated by a space of 4 in. stuffed with hay, and there was an air space of 4 in. between the inner shell and the glass sides of the tank. The lid was made in two halves, each consisting of a wooden frame supporting two glass plates separated by an air space of 3 in. A wooden cover above the lid carried two 40 W. electric bulbs at a height of 36 cm. above the surface of the water. The lamps were controlled by a clockwork time switch, and the fish received 12 hr. of light and 12 hr. of darkness each day.

Cambridge tap water was used throughout. The supply came from a constant-level tank set in a high window facing north, and it was distributed through a three-way junction (Fig. 1). One pipe led into a tank in which was immersed a refrigerating coil controlled by a thermostat. From this the water overflowed into a glass funnel and passed through a lagged pipe into the insulated tank *A*. The temperature of the water and its rate of flow could be controlled, and it was possible to maintain the temperature in this tank at any value between 4° C. and that of the room.

Another pipe led into a coil of metal tubing immersed in water in a dustbin lagged on the outside with felt; and from this coil, the water flowed into the insulated tank *B*. An electric immersion heater in the dustbin was controlled by a mercury-toluene thermostat in tank *B*, and the temperature of the water in this tank could be maintained at any value above that of the supply tank, which was about 14° C. in winter and rose to 17° C. in summer.

The third tank, *C*, was covered with a case of strong paper on a wooden frame, which carried two 40 W. electric bulbs. This tank could receive water either from the refrigerated tank or from the heated coil or directly from the supply tank. By combining these three sources it was possible to keep the temperature of the water at any desired value between the temperatures of the hot and cold supplies, and to alter it at any required rate. The temperature was recorded continuously by a thermograph.

All three tanks were fitted with overflow siphons which maintained constant water levels. The water in tanks *A*, *B* and *C*, in the refrigerated tank and in the dustbin, were stirred continually by compressed air.

The treatment of the fish in these tanks was exactly similar to that of the fish in the constant-temperature room at 11·5° C., as described in a previous paper (Brown, 1946*b*). They were weighed and measured generally once a fortnight, and individual records were kept for each fish. They were fed every day, except Sundays, with more meat than they would eat, and the excess food and faeces were siphoned out three times a week and dried. The dry weight was subtracted from the dry weight of food added, giving the amount of food absorbed by the fish. Trout were kept for at least 6 weeks under the experimental conditions before observations began. At the beginning of each experiment, 12 fish were placed in the tank and left to become acclimatized. At 18, 15 and 8° C., 12, 12 and 11 fish survived respectively, but at 4·5° C. only 9 survived, while at 20° C., after one unsuccessful attempt, 8 fish survived the whole period of the observations.

The environmental conditions in these tanks differed from those in experiments already described (Brown, 1946*b*) only in the temperature of the water. The fish were of the same stock and age, and the duration and intensity of illumination, the rate of flow, aeration and chemical composition of the water, the quality and quantity of food and the amount of living space were the same as those of trout in the constant-temperature room at 11·5° C.

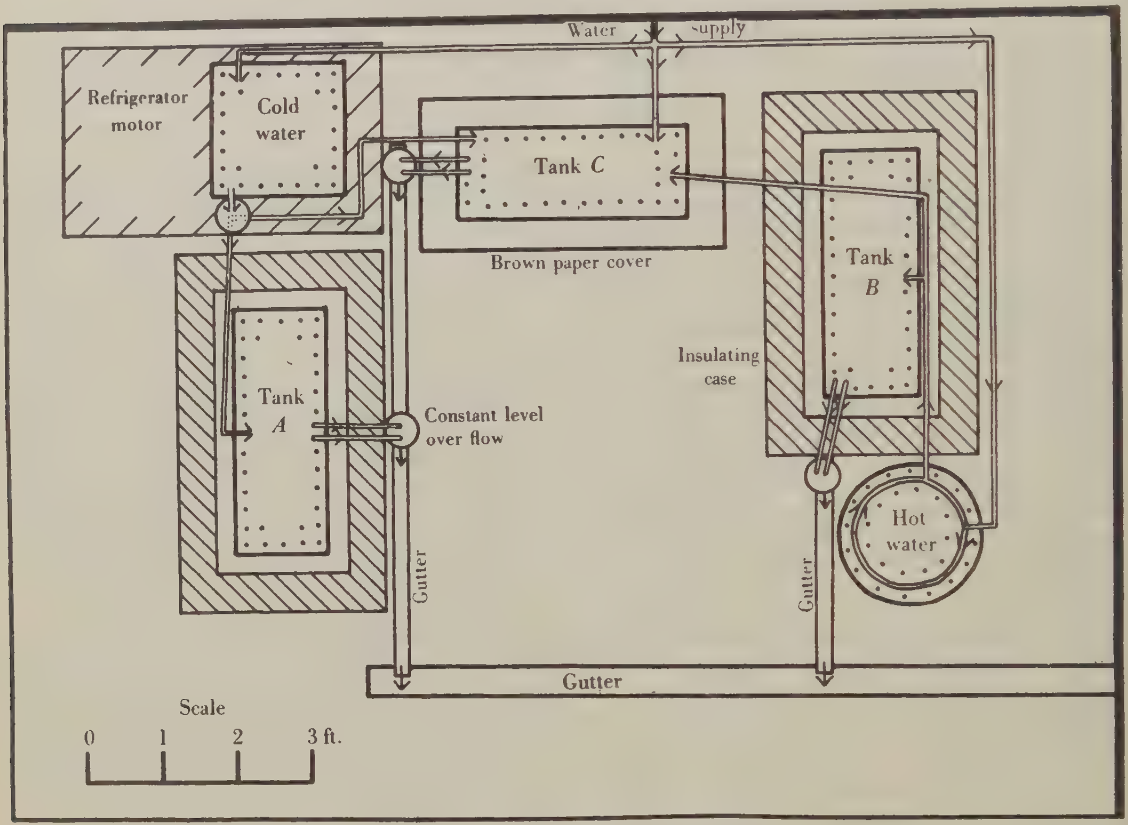


Fig. 1. Plan to show the arrangement of tanks and circulation of the water when trout were grown at different temperatures. *A*, tank with water temperature lower than that of the supply; *B*, tank with water temperature higher than that of the supply; *C*, tank with water temperature which could be adjusted to any value between that of the water in tanks *A* and *B*.

THE GROWTH OF TROUT AT DIFFERENT CONSTANT TEMPERATURES

Since there were only two insulating cases, tanks *A* and *B* were used successively to obtain the following temperatures: tank *A*, 4·5 and 8° C., and tank *B*, 15, 18 and 20° C. Fish living in water of constant temperature have an annual growth-rate cycle (Brown, 1946*b*), and consequently it was not possible to compare the growth of fish at all these temperatures directly with each other, but each must be compared with fish of the same age growing at 11·5° C. There were differences between the growth rates of the individual fish in each group; these appeared to be associated with their positions in the 'size hierarchies', and to be comparable with the differences between individuals growing at 11·5° C. It is thus possible to compare the mean specific growth rates of all the fish in each tank and also the specific growth rates of fish having the same positions in the size hierarchies of different tanks. All the fish showed fluctuations of growth rate over periods of from 4 to 6 weeks, and these appeared to be related to the condition factor, *K*, as was observed at 11·5° C.; only the specific rates calculated from weight data have therefore been considered in detail.

Table 1. *Comparison of the mean specific growth rates (calculated from weight data) of trout living in water of different constant temperatures*

Temp. in °C.	4·5	8	11·5	15	18	20
Mean specific growth rate (as percentage weight per week):						
Fish aged 90–102 weeks	—	4·81 ± 1·00	2·81 ± 1·30	—	3·98 ± 2·00	—
Fish aged 114–126 weeks	2·13 ± 0·63	—	4·06 ± 1·80	2·85 ± 0·78	—	2·30 ± 1·26

Table 2. *Results of statistical tests of the significance of differences between the specific growth rates of trout at* 11·5° C. *and at other temperatures*

Temp. °C.	Tested by comparison of means		Tested by analysis of variance	
	P	*t*	*P*	*t*
4·5	< 0·01	2·9	—	—
8	< 0·01	4·1	—	—
15	0·05	2·0	0·04	2·3
18	0·1	1·6	0·03	2·4
20	< 0·05	2·2	< 0·01	4·3

The average condition factors varied very little, all the means lying between 1·04 and 1·10.

Fig. 2A shows the mean specific growth rates of fish aged 90–102 weeks at 8, 18 and 11·5° C. The average rate at the last temperature was lower than those at the other two (Table 1), while the variation in rate, as measured by the standard deviations from the means, was greatest at 18 and least at 8° C. Fig. 2B shows the mean specific growth rates of trout aged 114–126 weeks at 4·5, 15, 20 and 11·5° C. The mean rate at the last temperature was higher than those at the others, and the variation between individuals was also greater (Table 1). The six largest fish at 11·5° C. grew more rapidly than the corresponding individuals at the other temperatures, but the smaller fish grew at similar rates at 11·5, 15 and 4·5° C.

The significance of the difference between the growth rates of trout at 11·5° C. and at the other temperatures has been estimated by two statistical methods: first, by comparison of the means, with due allowance for the small size of the samples; and secondly, by analysis of variance, comparing the performance of fish in the same positions in the size hierarchies of different tanks. The results of these tests are given in Table 2. The mean specific growth rate of the fish at 8° C. was significantly higher and that of the fish at 4·5° C. was significantly lower than that of fish of the same age at 11·5° C. By analysis of variance, individuals at 11·5° C. grew significantly faster than those of corresponding positions in the size hierarchy of the tank at 20° C. and probably faster than those at 15° C. and more slowly than those at 18° C.

The mean growth rates of trout at all six constant temperatures are compared in Fig. 3. Growth at 4·5° C. was slow, with little difference between individuals; at 8° C. the average specific rate was much higher; above this the variation among individuals was greater, and the mean specific rate fell to a minimum at 15° C., while at 18° C. some individuals were growing very rapidly and others comparatively slowly; and at 20° C. the variation was less, and the fish grew less rapidly, on the average, than they did at 11·5° C. If trout survived at higher temperatures, the mean specific rates would probably be still lower.

It might be argued that the differences in specific growth rate at the different constant temperatures were the result of genetical differences between the individual fish used in the experiments. To test this possibility, 12 two-year-old trout were kept in the insulated tank *A* for 2 months at 5° C., then for 3 months at 12° C. and then for 4 months at 8° C. The fish were allowed as much food as they would eat, and were weighed and measured once a fortnight. The mean specific growth rates at each temperature have been expressed as a percentage of the mean rate of fish of the same age at 11·5° C. (Table 3).

In this experiment, growth was relatively more rapid at 8 than it was at 12 or at 5° C., and this confirms the deductions made from comparison of different groups of individuals growing at these constant temperatures. It is therefore probable that

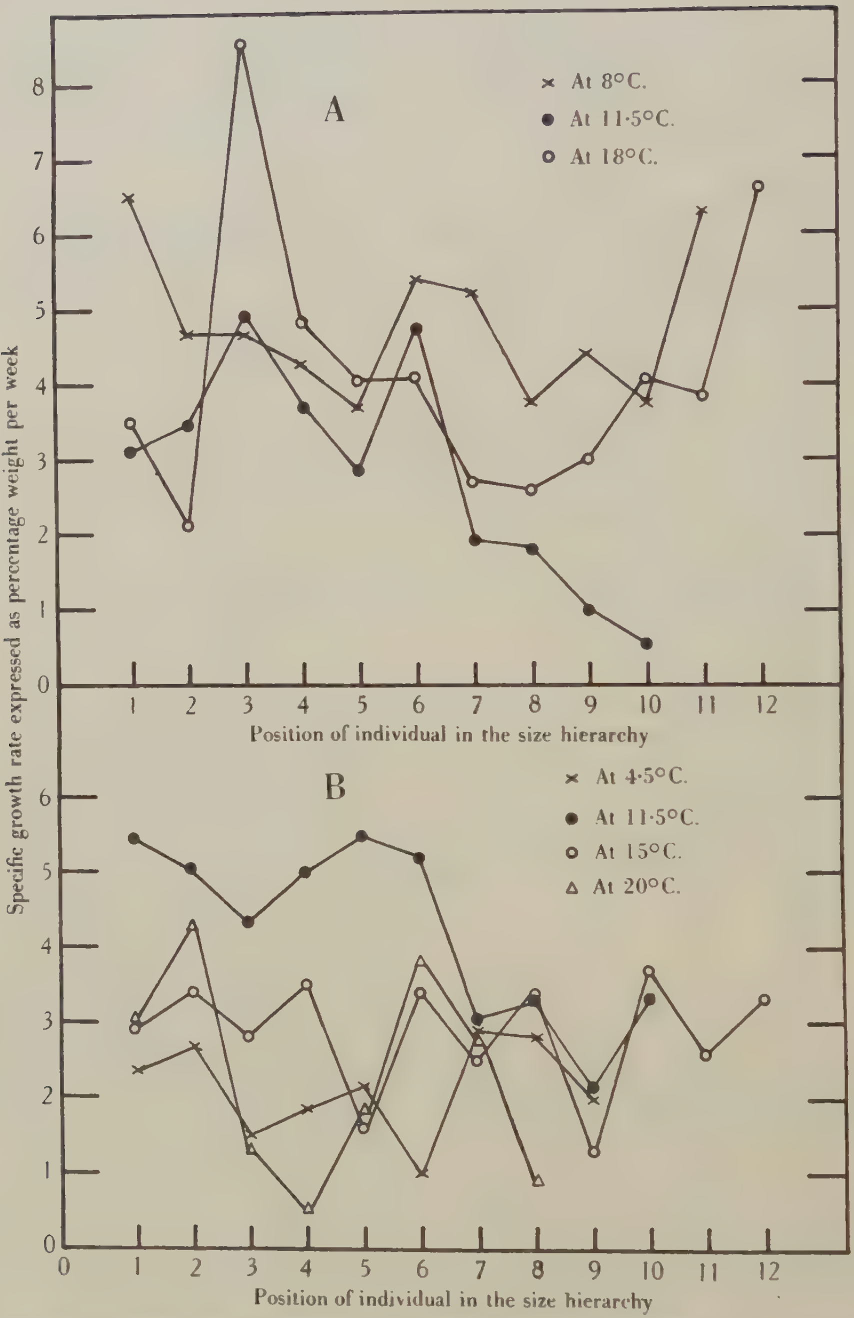


Fig. 2. Mean specific growth rates (calculated from weight data) of individual trout living at different constant temperatures. A, fish aged 90–102 weeks. Temperatures 8, 11·5 and 18° C.; B, fish aged 114–126 weeks. Temperatures 4·5, 11·5, 15 and 20° C.

the differences between the growth rates of groups of different individuals at constant temperatures are not the result of genetical differences between the individuals used for the experiments.

THE RELATION BETWEEN FOOD AND GROWTH AT DIFFERENT CONSTANT TEMPERATURES

The effect of temperature on maintenance requirements

When the trout had grown for a suitable length of time at the constant temperatures with as much food as they would eat, the amount given to them was reduced until their maintenance requirements could be estimated, using the technique described in the previous paper (Brown, 1946*b*). Using the values obtained in the experiments at 11·5° C., with fish of different weights, and assuming that the relationship between body weight and maintenance requirement is the same at all temperatures, the maintenance requirements of trout of average weight 50 g. have been calculated for each temperature (Table 4, Fig. 4).

The maintenance requirement is larger at higher temperatures, but the relationship is not direct. Below about 5° C. there may be a constant level of requirement; it increases gradually up to about 9° C., then rapidly up to about 11° C., and the rate of increase falls off gradually so that the requirement becomes nearly constant at 20° C. The temperature-maintenance requirement curve is thus sigmoid, with an inflexion at about 11° C.

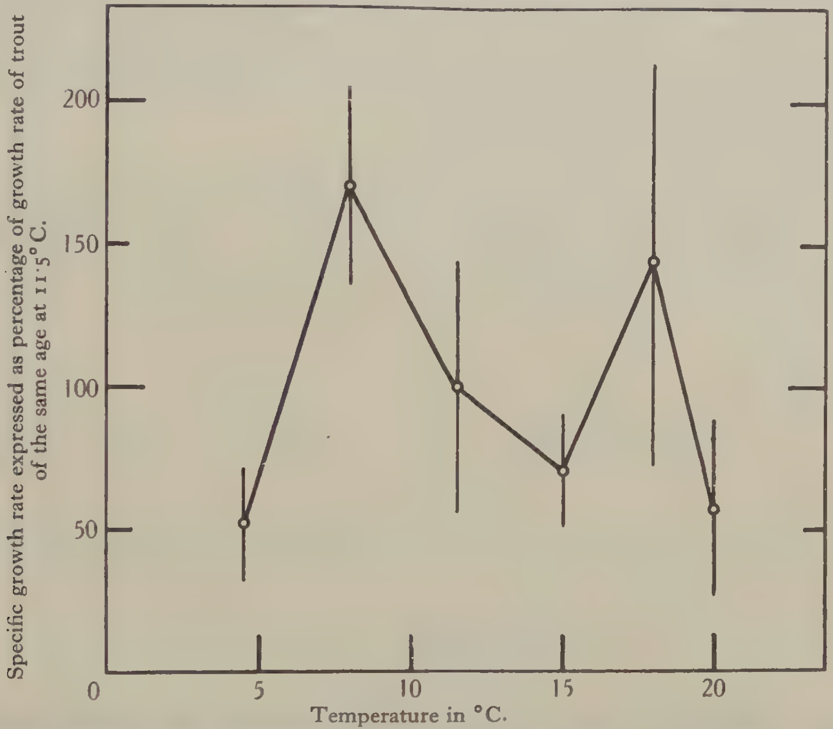


Fig. 3. Mean specific growth rates (calculated from weight data) of groups of trout living at different constant temperatures. The rates are expressed as a percentage of the growth rates of fish of the same age at 11·5° C. The height of the vertical lines = 2 × standard deviations from the means.

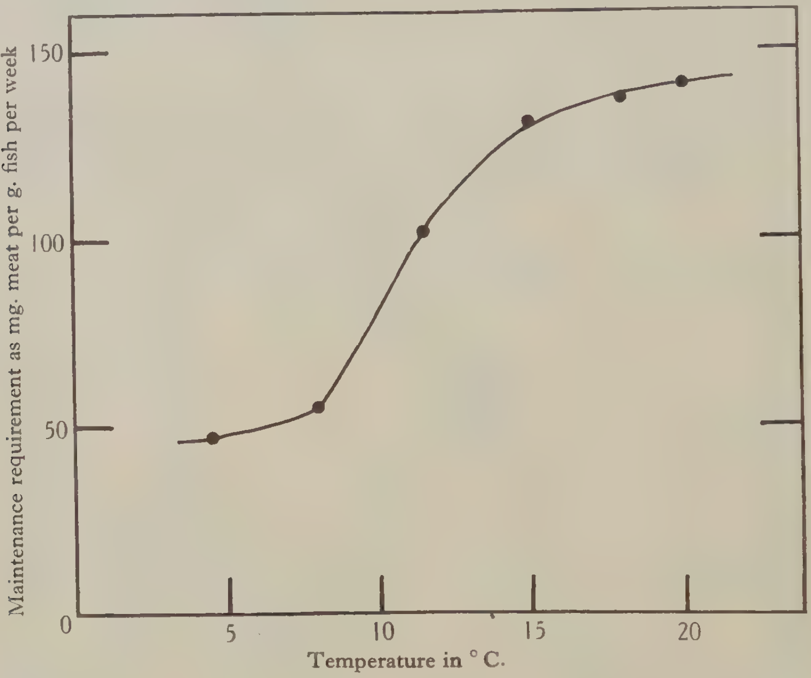
Table 3. *The mean specific growth rates (calculated from weight data) of a group of trout which were grown in succession at three different constant temperatures*

Age of trout weeks	Temp. ° C.	Mean growth rate expressed as a percentage of the growth rate of fish of the same age living at a constant temperature of 11·5° C.
118–126	5	42·4 ± 29
126–138	12	141 ± 55
138–154	8	192 ± 109

The effect of temperature on the amount of food eaten and the efficiency of utilization of food

The amount of food absorbed by trout when given as much meat as they would eat was determined for each fortnightly period at each temperature.

Maintenance requirements, calculated for fish of that average weight at that temperature, have been subtracted from these values, giving the amounts of food available for growth during each period. These values varied from week to week for each group, but showed no correlation with the average weight. Fish at 8° C. ate the greatest amount of food in excess of their calculated maintenance requirements, and the variation in amount was greatest at 8 and 18° C. and was very low at 20° C. (Table 5). There was markedly less food eaten in excess of the maintenance requirements at 5 and at 20° C. than at the intermediate temperatures.



To show the relation between the maintenance requirements calculated for fish of average weight 50 g. and temperature.

able 4. *Maintenance requirements (expressed as mg. per g. fish per week) of trout living at different constant temperatures*

Temp. t° C.	Average weight W g.	Maintenance requirement	Maintenance requirement of fish of weight W at 11·5° C.	Maintenance requirement of fish of weight 50 g. at t° C.
4·5	57·8	41	89	47
8	46·9	59	109	55·5
11·5	50	102	—	102
15	73·5	91	71	131
18	32·8	188	141	137
20	70·8	100	73	141

The average total amount of food absorbed by trout of average weight 50 g. at each temperature has been calculated by adding the maintenance requirement calculated for such a fish to the mean amounts of food eaten in excess of the maintenance requirements (Table 5). This amount is greatest at about 19° C. and decreases sharply above and gradually below this temperature. The change in total amount absorbed with rise in temperature is greatest below 8 and above 19° C., increasing at the former and decreasing at the latter temperature.

The efficiency of utilization of the food (that is, the change in average weight, expressed as mg. per g.

fish per week, divided by the amount of food eaten in excess of the maintenance requirement, also expressed as mg. meat per g. fish per week) was not constant from week to week at any temperature, but was correlated directly with the amount of increase in weight. The average efficiency was greatest at 8 and least at 4·5° C., and there was a minimum between 8 and 18° C. (Table 5). At 20° C., the average efficiency was high, although the specific growth rate was low.

Thus, efficiency and amount of food eaten were low below 8 and high at 8° C.; the growth rate minimum between 10 and 16° C. was associated with lower efficiency and a greater amount of food eaten; at 18° C. the efficiency was greater and relatively more food was eaten in excess of the maintenance requirements; while at 20° C. growth was limited by the small amount of food eaten, since the efficiency was high.

Table 5. *The amount of food eaten and the efficiency of utilization of food of trout at different constant temperatures*

Temp. ° C.	Amount of food eaten in excess of maintenance requirements (mg. meat per g. fish per week)	Total amount eaten by fish of average weight 50 g. (mg. meat per g. fish per week)	Efficiency of utilization
4·5	115 ± 31	160	0·12
8	174 ± 67	225	0·28
11·5	159 ± 44	260	0·22
15	146 ± 38	275	0·20
18	147 ± 54	280	0·24
20	102 ± 14	240	0·24

THE FEEDING AND GROWTH OF TROUT IN A CHANGING TEMPERATURE

The specific growth rate of trout in water with a changing temperature

Twelve fish were grown in tank C (Fig. 1), which could be supplied with either warm or cold water so that its temperature could be controlled between the limits of 4 and 20° C. The fish were given as much food as they would eat, and they were weighed and measured once a fortnight. The temperature was first reduced from 18 to 4° C., at the rate of ½° C. per week; it was then raised to 18° C. at the same rate; after 8 weeks at this temperature, it was reduced to 4° C. again, but at the rate of 1° C. per week, and then increased to 20° C. at the same rate. All the fish survived for 1 year in this tank, but seven fish died when aged 150–158 weeks, when the temperature was 18° C., and they were found on dissection to have enlarged gonads. The surviving fish, all males, lived until the end of the observations.

Mean monthly specific growth rates have been calculated for this group of fish, and these rates have been expressed as a percentage of the mean specific rates of fish of the same age living at a constant temperature of 11·5° C. The mean temperature has been calculated for each month. The range of temperature is 2° C. for each month for fish aged 90–150 weeks, and 4° C. for each month for fish aged over 158 weeks, so the correlation between mean growth rate and average temperature is necessarily an approximation. This correlation is shown in Fig. 7.

There is a growth rate maximum at a temperature above 14° C. in all records except that for fish aged 170–194 weeks, when the temperature was rising at the rate of 1° C. per week; there is a maximum at a temperature between 7 and 9° C. in all the records except that for fish aged 90–120 weeks, when the temperature was falling at the rate of ½° C. per week. The mean specific rates were the same as those of fish of the same age living at a constant temperature of 11·5° C. at a temperature between 9 and 12° C. in all the records. Fish living in a tank with the water temperature changing gradually at a constant rate thus showed approximately the same relationship between temperature and growth rate as fish living at different constant temperatures. The growth rate maximum below 10° C. was generally not so great as that above 15° C. in the changing temperature, and there was no indication that rising and falling temperatures had different effects on growth.

The amount of food eaten and efficiency of utilization of fish in a changing temperature

The amounts of food eaten in each period by the two-year-old trout have been calculated, the estimated maintenance requirements have been subtracted, and the amounts eaten in excess of the maintenance requirements have been correlated with the average temperature (Table 6). These amounts are smaller, and decrease more markedly above the optimum temperature, than those eaten by fish at corresponding constant temperatures. The average efficiency of utilization of the food is also comparable with that of fish at different constant temperatures (Table 6), but the value for 7–10° C. is lower and that for 16–19° is higher.

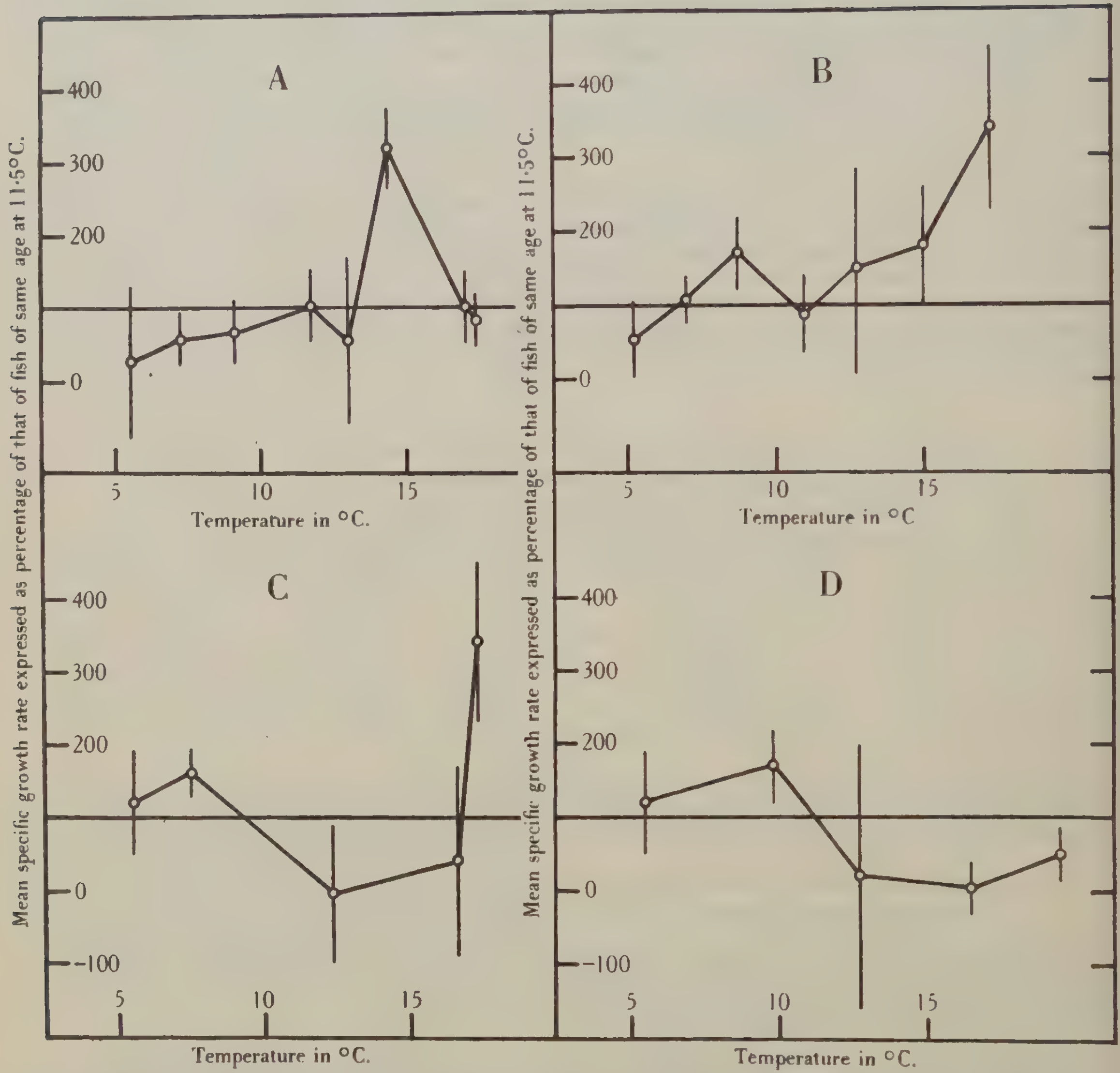


Fig. 5. Mean monthly specific growth rates of a group of trout living in water with a changing temperature. The rates are expressed as a percentage of the growth rates of fish of the same age at 11·5° C. The height of the vertical lines = twice the standard deviations from the means. A and B, with the water temperature changing at the rate of ½°C. per week: A, temperature falling, fish aged 90–120 weeks; B, temperature rising, fish aged 120–158 weeks. C and D, with the water temperature changing at the rate of 1°C. per week: C, temperature falling, fish aged 150–174 weeks; D, temperature rising, fish aged 170–194 weeks.

Fish in a changing temperature thus show a similar relationship between food, growth and temperature to that shown by fish living at different constant temperatures, except that under the former conditions the amount of food eaten was relatively smaller and a given change in weight occurred with greater efficiency than at the corresponding constant temperatures.

Table 6. *The amount of food eaten and the efficiency of utilization of food of trout living in water of changing temperature*

Mean temp. ° C.	Amount of food eaten in excess of maintenance requirements (mg. meat per g. fish per week)	Efficiency of utilization
4–7	67	0·17
7–10	120	0·24
10–13	102	0·18
13–16	77	0·24
16–19	58	0·33

DISCUSSION

Temperature affects the growth rates of animals by altering the rates of various metabolic processes, and its effects are likely to be more marked if the animals are poikilothermic than if they are homoiothermic. Increase in temperature increases the respiratory metabolism of fish (Gardner & Leetham, 1914; Gardner, 1926; Wells, 1935; Clausen, 1936; Sumner *et al.* 1938, 1942), and there is an increase in the maintenance requirements (Dawes, 1930; Pentelow, 1939), and more rapid loss of weight during starvation (Pentelow, 1939; Lawrence, 1940). Increase in temperature also increases the activity of fish, leading to increased food intake (Hathaway, 1927), and there is increase in the rate of digestion (Scheuring, 1928; Hewitt, 1943) and the efficiency of conversion of food into fish may be altered (Hayes & Pelluet, 1945).

The effect of increase in temperature is thus to increase the amount of food required for maintenance and also to increase the amount eaten by the fish. The rate of growth will vary with the ability of the fish to digest more food than is required for maintenance at that temperature, and it is probable that, with increase in temperature, the growth rate will increase to a maximum and then decrease, becoming negative at high temperatures. This relationship between temperature and growth rate was found by Audigé (1921) for *Cyprinus*, *Carassius* and *Scardinius*, with an optimum at 25° C.; by Pentelow (1939) for *Salmo trutta*, with an optimum between 10 and 15° C.; by Donaldson & Foster (1940) for *Onchorhyncus*, also with an optimum between 10 and 15° C.; and by Hewitt (1943) for *Salmo trutta*, with an optimum between 15 and 19° C. Wingfield (1940) suggested that rising and falling temperatures have different effects on the growth rate of yearling trout.

The effect of temperature on the embryonic growth of *Salmo fario* illustrates the same principles (Gray, 1928; Wood, 1932). The time required for complete development of the embryo was less at higher temperatures, but between 7 and 12° C. the total amount of oxygen consumed during development was the same at all temperatures, and the resulting fry were of equal size. Above and below these temperatures, the total amount of oxygen consumed was greater and the fry were smaller.

The experiments described in the present paper indicated two temperature optima for rapid growth of two-year-old brown trout, one between 7 and 9° C. and the other between 16 and 19° C., and these optima occurred when fish were grown in a changing temperature as well as at different constant temperatures. The maintenance requirements increased, relatively, with increase in temperature, but the curve was sigmoid. The requirement as estimated included not only the amount of food required for basal metabolism (i.e. to replace tissues broken down and to provide energy for the fish to live while at rest) but also the amount required to provide energy for locomotion. The basal metabolic rates of animals increase with rise in temperature, the rate of increase generally rising with the temperature. Since the observed relation between temperature and maintenance requirement followed a sigmoid curve, it is suggested that the activity of the fish rose to a maximum at 10–12° C. and decreased above this temperature; the reduction of activity at the higher temperatures was sufficiently great to compensate partially for the increase in basal metabolism.

The amount of food eaten was at a maximum between approximately 10 and 19° C., so that the upper optimum temperature (18° C.) coincided with a large amount of food eaten and low activity, and the slower growth above 19° C. may be explained by the smaller amount of food eaten and increased basal metabolic rate (Fig. 6). At 5° C. both activity and amount of food eaten were small, while at 8° C. both were higher, but the basal metabolic rate was still low. Between 10 and 15° C. the amount of food eaten and activity were both maximal, and the basal metabolic rate had increased. Thus, the lower optimum temperature coincided with a maximum in the amount of food eaten in excess of the maintenance requirement. Thus it is possible to explain the existence of two optimum temperatures for trout growth by postulating a differential effect of temperature on the amount of food eaten and the activity of the fish (Fig. 6).

The efficiency of utilization of the food also varied with the temperature, being low at 5, high at 8 and above 17° C. and low between these two temperatures. Thus, the efficiency was low when the temperature was low and also when the activity was high (Fig. 6). This would exaggerate the growth rate maxima and minima.

Elson (1942) measured the activity of *Salvelinus fontinalis* in response to an electric shock at different temperatures, and found that it was greatest at 10° C. and decreased above and below this temperature. Wells (1935), using *Fundulus parvipinnis*, noted that increase in temperature was accompanied by increase in oxygen consumption, but the temperature-respiration curve contained sudden breaks at definite temperatures, and he suggested that these were correlated with changes in the degree of activity of the fish. Gardner & Leetham (1914) and Gardner (1926) found that the oxygen consumption of brown trout was doubled for the 10° rise between 8 and 18° C., but their curves are not sufficiently detailed to show whether sudden changes in consumption occurred. Thus, it is not incompatible with other work that trout should have a maximum degree of activity between 10 and 12° C.

Pentelow (1939) and Wingfield (1940) correlated the growth of their trout with the average monthly temperatures of their tanks. In neither series of experiments was the temperature controlled, and in some months there were probably large fluctuations, so the temperature-growth correlations were necessarily approximate. Both authors deduced that the rate of growth was maximum between 10 and 15° C. There were few records for temperatures higher than 15° C., and half their observations for the temperature range 7–9° C. were made in the autumn. At this season, trout living in environments of constant temperature showed a decrease in their specific growth rates (Brown, 1946*b*), and Pentelow's table correlating temperature and growth (1939, p. 458) contains almost equal numbers of records of large and small changes in weight for temperatures between 46 and 50° F. Wingfield found that when the temperature was falling (i.e. during late summer and autumn) the growth rate for any given temperature was less than when it was rising (i.e. spring), and his fish showed increase in their growth rate in February, although there was no increase in water temperature. These aberrations can be explained by the existence of an annual growth-rate cycle.

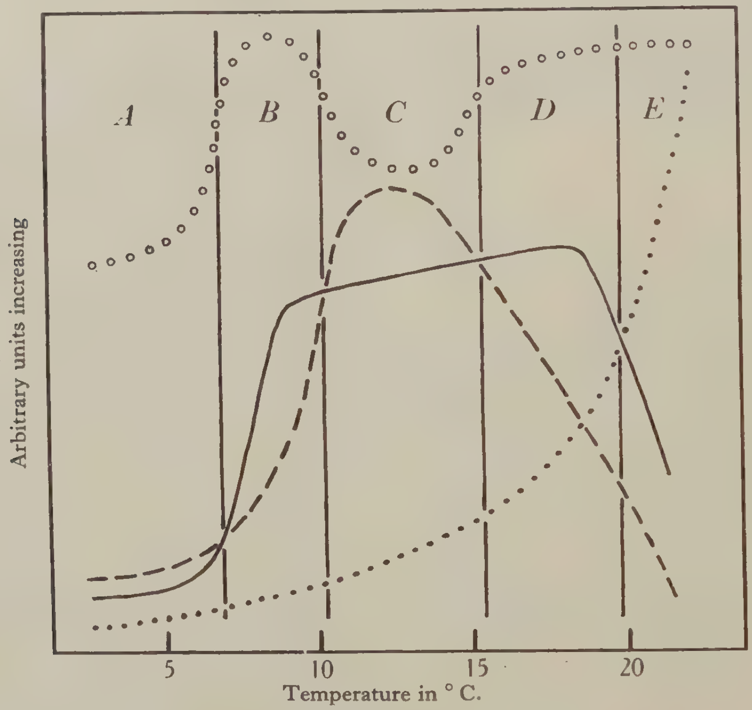


Fig. 6. Diagram to illustrate how growth rate maxima at two different temperatures could result from a differential effect of temperature on the amount of food eaten (full line), the activity (broken line) and the basal metabolic rate (dotted line) of trout. The vertical lines divide the whole temperature range into five regions. Within regions *A*, *C* and *E*, the specific growth rates would be low; within regions *B* and *D*, the rates would be high. The change in efficiency (circles) with change in temperature would exaggerate these growth rate minima and maxima.

Pentelow (1939) measured the maintenance requirements of trout and found that they increased with increase in temperature. The majority of his determinations were performed at temperatures between 46 and 50° F., when the majority of values were between ·70 and 102 mg. *Gammarus* per g. fish per week, the average weight of the fish being about 20 g. The requirement estimated during the experiments described in the present paper was 55 mg. meat per g. fish per week for fish of average weight 50 g. at 8° C. Since the requirements decrease, relatively, with increase in weight, the agreement between these two estimates is good.

Pentelow (1939) found that yearling trout required 3·0 g. of *Gammarus* more than the maintenance requirement for an increase in weight of 1·0 g. In the experiments described in this paper, two-year-old fish with maximum rations at 8° C. required 3·6 g. of meat more than the maintenance requirement for an increase of 1·0 g. in weight. If the difference between these two estimates is significant, either *Gammarus* is used more efficiently than meat during trout growth, or the fish use food less efficiently as they grow older.

SUMMARY

1. Two-year-old trout were grown in controlled environmental conditions in water of different temperatures.

2. The specific growth rates of trout living at different constant temperatures and of those living in water of changing temperature were high between 7 and 9° C. and between 16 and 19° C., and were low above, between and below these temperatures. The existence of these two growth rate maxima may be explained by a differential effect of temperature on the amount of food eaten and the activity of the fish, the former being maximal between 10 and 19° C. and the latter between 10 and 12° C. The efficiency of utilization of the food was low when the temperature was low and also when the activity was high.

3. The maintenance requirements of trout of equal weight increased with increase in temperature. The relation followed a sigmoid curve, which may be explained by a differential effect of temperature on the basal metabolism and on the activity of the trout.

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THE DIETETICS OF THE CLOTHES MOTH, *TINEOLA BISSELLIELLA* HUM.

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(With Seven Text-figures)

Much attention has been paid in the past to the peculiar mechanism of keratin digestion which enables the clothes moth to infest woollen materials (Schulz, 1925; Linderstrøm-Lang & Duspiva, 1935; Duspiva, 1936), but little is known about its general food requirements. It is known that the larvae will not grow on clean wool (Titschak, 1922; Crowell & McCay, 1937) and that damage to clothing is generally restricted to parts soiled by dirt and sweat. On the other hand, *Tineola* has been bred in the laboratory on various foods in the absence of keratin, and there are no indications that keratin is a necessary constituent of the food (Titschak, 1922). A study of some aspects of the nutrition of *Tineola* has been made by Crowell & McCay (1937) and will be discussed below.

METHODS

The methods employed are similar to those described in earlier publications (Fraenkel & Blewett, 1943*a, c*). Ten larvae were always grown together in a 2 × 1 in. specimen tube containing 2 g. of food, and each test was done in duplicate. All the tests were performed at 25° and 70% relative humidity. They were started with the first stage larva and concluded with the emergence of the adult moth. *Tineola* as a rule pupates inside the food and therefore pupation cannot be observed. The pupal period is most probably constant and independent of the nature of the larval food, and it lasts, at 25° and 70% R.H., about 10 days.

General food requirements

Tineola grows well on an artificial food mixture composed of

Casein (A/E, Glaxo Lab.)	50 parts
Glucose	50 ,,
Yeast (brewers', debittered, Glaxo Lab.)	5 ,,
Cholesterol	1 part
McCollum's salt mixture	2 parts
Water	10 ,,

On this food, called 'glucose diet' in Table 1, at 25° and 70% R.H., adult moths start to emerge after about 33 days, and most adults emerge between the 33rd and 39th day. As the pupal stage lasts about 10 days, the larval period is about 25 days. This growth rate, as will be shown later, compares very favourably with the speed of growth on other, more natural, food substances and is considerably faster than that reported for larvae on woollen materials. Glucose is not an essential constituent of this diet; if the same amount of casein is substituted for it, the food remains in every respect as efficient (Table 1).

Table 1. *Emergence of* Tineola bisselliella *on various diets. Total number of moths formed by the 31st, 33rd, etc., day. Twenty larvae in each experiment*

Days ...	31	33	35	37	39	41	43	45
Glucose diet*	—	3	11	11	18	18	19	—
Same, plus wheat-germ oil	1	4	8	8	14	14	15	—
Diet without carbohydrates	—	2	6	6	11	14	17	—
Glucose diet plus wool fibres	—	—	8	16	16	16	18	20
Glucose diet with ether-extracted yeast	—	1	8	10	15	17	18	—
'All vitamins' diet†	—	—	6	8	9	15	19	—
'All vitamins' diet plus yeast fat	—	—	3	9	9	10	16	17

* Composition given on this page.
† Composition given on p. 157.

If glucose is replaced by starch (maize), growth is retarded by about 10 days (Fig. 4). It is therefore evident that a carbohydrate is not required in the diet and that the presence of starch is somewhat detrimental. Without cholesterol no growth takes place and the larvae die at an early stage. Yeast is equally an essential constituent of the diet; no growth takes place if yeast is omitted. There are no indications that addition of wool improves the efficiency of the diet (Table 1). Addition of a fat (wheat-germ oil) does not alter the growth rate (Table 1).

Requirements of vitamins of the B group

An investigation of the importance of the water-soluble vitamins, contained in yeast, was made by the method described in an earlier publication (Fraenkel & Blewett, 1943*c*). Instead of yeast a mixture of pure vitamins of the B group was given and in addition the diet contained 5 parts of 'insoluble' yeast (yeast exhaustively extracted with boiling water to remove water-soluble constituents). The diet then consisted of

			µg./g. of dry diet
Casein	50 parts	Thiamin (B_1)	50
Glucose	50 ,,	Riboflavin	50
'Insoluble yeast'	5 ,,	Nicotinic acid	50
Cholesterol	1 part	Pyridoxin (B_6)	50
Salt mixture	2 parts	Pantothenic acid	50
Water	10 ,,	Inositol	500
		Choline chloride	500
		p-Amino-benzoic acid	10

On this diet, called subsequently 'all vitamins' diet in Fig. 1 and in Table 1, growth was in every respect as good as on the original diet, which contained 5 parts of yeast. Leaving out single vitamins of the B complex, one at a time, affected the efficiency of the diet to a different degree with different vitamins (Fig. 1). Growth was equally good with or without inositol or *p*-amino-benzoic acid. Growth became increasingly slower if either riboflavin, choline, pyridoxin or thiamin were omitted, but most larvae completed development. In the absence of pantothenic acid growth was slower still, and only three out of twenty reached the adult stage, while no growth took place in the absence of nicotinic acid.

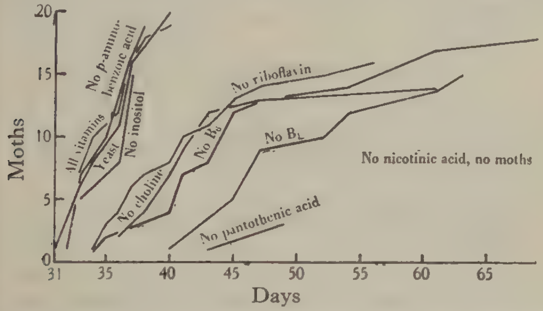

Fig. 1. Growth of *Tineola bisselliella* on an artificial diet, described above, which contains the known members of the vitamin B complex in pure substance ('all vitamins') compared with similar diets from which single vitamins have been left out, one at a time.

In view of the fact that *Tineola* eventually pupates in the absence of any one of the B-group vitamins, except nicotinic acid, an attempt to determine the minimum optimal requirements was only made with nicotinic acid. A graded growth response was obtained with graded doses of nicotinic acid, and growth was optimal with 8–16 per g. of the diet (Fig. 2).

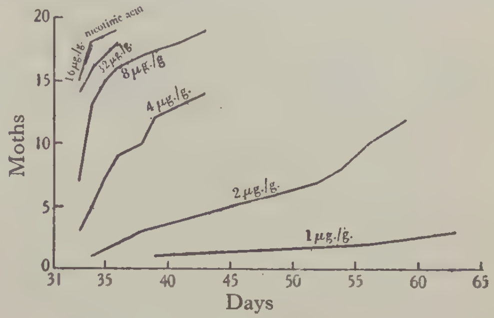

Fig. 2. Growth of *Tineola bisselliella* on a nicotinic acid-free diet with the addition of graded doses of nicotinic acid.

With all the B vitamins present in the diet, but without the insoluble part of yeast, growth was very slightly retarded. Addition of biotin (0·1 µg./g.) to this diet possibly somewhat improved it (Fig. 3). The following vitamins are therefore of some importance in the nutrition of the *Tineola* larva: thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, choline and possibly biotin.

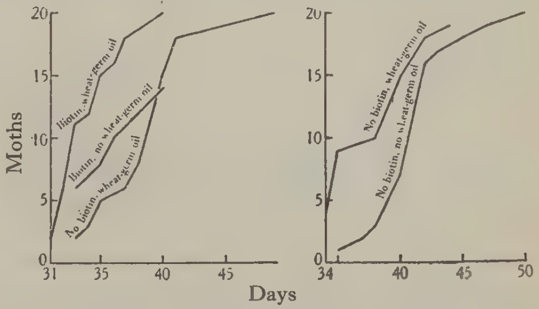

Fig. 3. Growth of *Tineola bisselliella* on artificial diets in the presence or absence of biotin or wheat-germ oil.

Fat requirements

The artificial diet described on p. 156 contains no fat, apart from such small amounts which the yeast contained. Extracting the yeast with ether has not the slightest effect on the efficiency of this diet (Table 1). This is not a conclusive result, as a Soxhlet extraction might not remove all traces of fat. There was, furthermore, no evidence that addition of fat, neither wheat-germ oil nor the fat extracted from yeast with petrol ether, improved the diet (Table 1). It may be argued that in all these diets very small amounts of fat were supplied with yeast, and therefore a further comparison was made using diets which did not contain yeast or 'insoluble yeast' and where the B vitamins were supplied in pure substance (Fig. 3). On this diet, both in the presence and absence of biotin, growth was slightly speeded up by the addition of wheat-germ oil. This indicates that some fat-soluble substance may be of slight advantage for the development of *Tineola bisselliella*.

Growth on flour and other natural food substances

The ease with which the clothes moth can be grown on mixtures of pure food substances made it probable that some dried foods might also constitute an adequate food. In fact, an unwanted infestation of certain dried foods, stored in the laboratory, e.g. yeast and fishmeal, has frequently occurred with us. Results of the breeding of *Tineola bisselliella* on several dried foods are shown in Figs. 4 and 6. On flours of different extraction the speed of development is dependent on the rate of extraction and falls as the extraction is lowered (Fig. 4). This phenomenon, which has often been demonstrated with other insects (e.g. Fraenkel & Blewett, 1943*b*), is almost certainly mainly due to the smaller amounts of vitamins of the B group in low extraction flour. Even wholemeal flour is not an optimal food and can be greatly improved by adding 5% yeast. In attempting to find out whether a single factor is responsible for this deficiency in wholemeal flour, tests were carried out with wholemeal flour to which riboflavin or nicotinic acid was added. Riboflavin was chosen, because wheat is relatively short of this factor, and nicotinic acid, because *Tineola* appears to require this factor most. There was certainly an improvement with nicotinic acid, but not with riboflavin (Fig. 5).

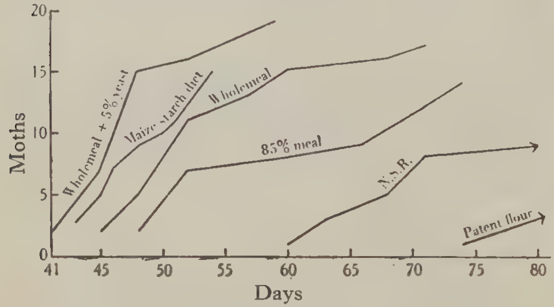

Fig. 4. Growth of *Tineola bisselliella* on flour of different rates of extraction (wholemeal = 100%, National Flour = 85%, National Straight Run = 72%, Patent Flour = 40%) and on an artificial diet which contains starch.

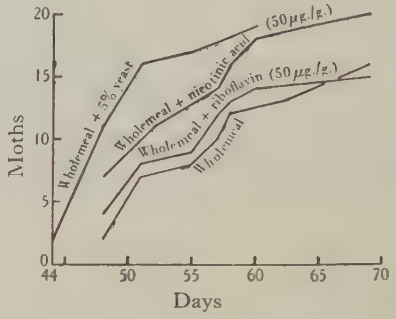

Fig. 5. Growth of *Tineola bisselliella* on wholemeal flour with the addition of riboflavin or nicotinic acid.

Tineola also grows successfully on fishmeal, meatmeal, wheat germ and yeast, but in no case was development as fast as on our artificial diets. Fishmeal again was greatly improved by the addition of 5% yeast (Fig. 6).

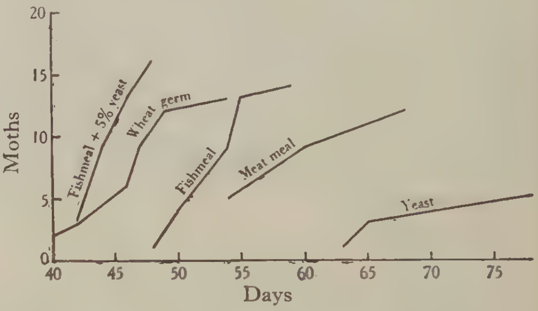

Fig. 6. Growth of *Tineola bisselliella* on different natural foods.

Growth at different relative humidities (Fig. 7)

The rate of growth of the clothes moth larva is greatly dependent on the relative humidity to which the diets are exposed. As in the case of many other insects (e.g. Fraenkel & Blewett, 1943*b*, 1944*b*) growth is faster at high humidities. But even at 30% R.H. the majority of the larvae ultimately complete de-

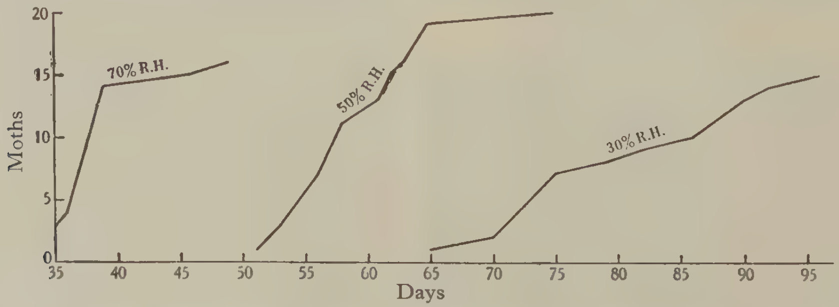

Fig. 7. Growth of *Tineola bisselliella* on an artificial diet described on p. 156, at different relative humidities.

velopment, though after a long delay. This agrees well with results by Griswald & Crowell (1936), who found that even at 20% R.H. twenty-four moths emerged out of a total of thirty larvae.

DISCUSSION

An analysis of the nutritional requirements of the clothes moth larva has been attempted once before (Crowell & McCay, 1937). These authors found that a supply of B vitamins, given in the form of a 'Harris B concentrate', was essential for growth. Purified casein appeared to be a not entirely adequate protein, but was satisfactorily supplemented by purified lactalbumin. The value of ash (from fishmeal) was doubtful. On the most successful diet consisting of 89·5% casein, 10% lactalbumin and 0·5% B concentrate the life cycle averaged 48·2 days, as compared with 55 days on fishmeal. This is considerably slower than growth on our best diets, although the temperature of the experiments was the same—25°. In the light of our present knowledge it seems most probable that Crowell & McCay's diet could have been improved by the addition of a sterol. As the casein and lactalbumin in that diet were 'vitamin-A free', one would expect them to be relatively free of sterol also. The diet must, however, have contained some sterol, possibly in the yeast concentrate, otherwise growth would have been impossible. In some of our diets casein was the sole source of protein and proved entirely satisfactory. This makes it improbable that the beneficial effect obtained by the addition of lactalbumin was due to a protein deficiency and suggests that the lactalbumin might have contained some impurity of importance.

In its food requirements, the clothes moth larva in general resembles other insects recently investigated by us (Fraenkel & Blewett, 1943*a*, *c*). Like other insects, it requires a sterol, and the amount of sterol present in 5% yeast proved insufficient. It also resembles other insects in its requirements for vitamins of the B group. The five factors of the B complex, thiamin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid, which most insects seem to require, are also of importance in the nutrition of the clothes moth. But in contrast to the needs of certain other insects, only nicotinic acid and pantothenic acid proved indispensable. In the absence of any of the other B vitamins growth was delayed, but did not stop. In this the clothes moth resembles *Lasioderma* and *Sitodrepa*, which by virtue of the presence of intracellular symbiotic yeasts have become somewhat independent of the extraneous supply of B vitamins (Fraenkel & Blewett, 1944*a*). No intracellular symbiosis has, however, ever been reported from a lepidopter, and a cursory examination of the gut system of the clothes moth larva has not so far revealed the presence of either intracellular symbionts or of any conspicuous micro-organism in the lumen of the gut to which the production of B vitamins might be attributed. None the less, for optimal growth, most factors of the B complex are required. Even such a good source of B vitamins as wholemeal flour is not an optimal diet and is considerably improved by the addition of 5% yeast. It is interesting to note that the presence of riboflavin has been demonstrated in the Malpighian tubes of clothes moth larvae which had been grown on a food free of riboflavin (Busnel & Drilhorn, 1943).

The clothes moth, like all the other insects so far investigated, does not require inositol or *p*-aminobenzoic acid. In the presence of all the eight chemically known factors of the B complex growth is as good as on a diet which contains yeast. This shows that no further soluble or insoluble factors in yeast are required.

Particular effort has been made to find out whether any fat-soluble factor is required in addition to sterol. The search for such fat-soluble factors has so far proved unsuccessful in other insects, except in the case of caterpillars of the genus *Ephestia*, which in the absence of linoleic acid and vitamin E or some other unsaponifiable substance fail to emerge from the pupal case (Fraenkel & Blewett, 1945). Most of our tests suggested that no fat is required. In only two cases addition of fat proved very slightly beneficial (Fig. 3), and this occurred in diets which contained no yeast and where the presence of traces of fat was excluded. This makes it possible that in all the other diets yeast might have supplied the necessary fat. On the other hand, fat improved growth only very slightly, and there were no indications that the absence of fat had an unfavourable effect on the emergence of the adult as in the case of *Ephestia*.

The principal food of the clothes moth is generally considered to be woollen cloth. The main clue to this particular food habit is the ability of this insect to digest keratin. That pure keratin cannot serve as a satisfactory food has been shown again and again, and it would be very surprising indeed if it were otherwise. It is now well recognized that growth is only possible on cloth soiled by dirt, sweat or urine. Even so, it is astonishing enough that any higher organism can develop on a food of such poor quality. We recognize now at least five factors which go far to make the peculiar food habits of the clothes moth understandable: (1) its extraordinary ability to digest keratin (Duspiva, 1936; Linderstrøm-Lang & Duspiva, 1935), (2) its relative indifference to the absence of certain vitamins of the B complex, (3) its ability to grow under very dry conditions, e.g. at 20% R.H., (4) its indifference to the presence of a carbohydrate, and (5) of a fat in the diet. Even so the clothes moth grows only relatively slowly on cloth, very much slower than on our artificial diet and several other foods. This may be due to a number of factors. Although keratin is digested it may possibly not constitute an optimal source of amino acids. In experiments with rats it has been recently shown that keratin, under certain conditions, can serve as a source of protein, if supplemented by tryptophane, methionine, histidine and lysine, in which wool is low (Anon. 1944). With regard to B vitamins the supply may not necessarily be too unfavourable. As already mentioned clothes moths mostly feed on places soiled by sweat or urine. It has been known for long that water-soluble vitamins are excreted with the urine. Recently (Anon. 1943) it has also been shown that vitamins of the B complex, namely thiamin, riboflavin, nicotinic acid and pantothenic acid, are lost in sweat. The amounts concerned are small in relation to daily requirements, but it is conceivable that significant amounts would be concentrated over long periods on certain parts of a garment.

Of foods which we have tested our artificial diet allows the fastest development to take place, but a number of other foods have proved favourable breeding grounds for *Tineola*. Of these, wholemeal flour and fishmeal are obviously deficient in certain B vitamins, as is indicated by the improved growth rate after 5 % yeast has been added to these foods. On flours of different extraction, duration of development and mortality increases as the extraction is lowered. This has been shown already for a number of other insects (Fraenkel & Blewett, 1943*b*), and is explained by the smaller amounts of B vitamins in white flour. But even on wholemeal flour, supplemented with 5 % yeast where the concentration of B vitamins could be expected to be optimal, growth is much slower than on our best artificial diets. This is most probably due to the presence of large quantities of starch in flour. In an artificial diet, growth is much slower if starch is used instead of glucose, and about equal to that of flour plus yeast (Fig. 4).

Although flour and other dried foods tested by us are not optimal diets, it still remains unexplained why the clothes moth does not commonly become a pest on them, as development is even slower on cloth. According to Titschak (1925), total development on dirty woollen cloth, at 25°, takes on the average 71·9 days for males and 89·1 days for females. But damage to clothing may be caused by only a few growing larvae, whereas on food a much heavier infestation must develop before noticeable damage occurs. It may be suggested that in temperate climates the temperature would be too low to allow development of large numbers of clothes moths in stored foods. This is certainly not the case. At 15° the life cycle takes about 190 days on cloth (Titschak, 1925), and we can infer from this that on flour at the same temperature the life cycle would take about 120 days. Larvae survive very cold winter temperatures (Mansbridge, 1936), and though development stops below 5° (Yamada, 1940), it is resumed when the temperature rises in the spring (Zacher, 1933). One would therefore expect that, given sufficient time, probably over a year in southern England, large numbers of clothes moths could develop and ultimately become a pest on certain dried foods. In fact, on some occasions, clothes moths have been reported as pests on stored grain (Austen & McKenny Hughes, 1942; Cotton & Good, 1937), and during the present war outbreaks have occurred on stored flour.

Our investigations suggest that relative to the populations of such moths as the *Ephestia* species, for example, those of *Tineola* and its fellow clothes moths are small and that the economic importance of the clothes moth is very high relative to the size of populations and to the amount of food they consume.

SUMMARY

1. The larva of the clothes moth *Tineola bisselliella* Hum. grows well on an artificial diet consisting of casein, glucose, yeast, cholesterol, salt mixture and water. The presence of glucose is unnecessary, and that of cholesterol and yeast necessary for growth.

2. Yeast has been successfully replaced by a mixture of eight pure vitamins of the vitamin B complex. In the absence of either riboflavin, choline chloride, pyridoxin or thiamin growth is retarded, while little or no growth takes place in the absence of pantothenic acid or nicotinic acid. Inositol and *p*-amino-benzoic acid do not seem to be necessary, and the value of biotin for growth is doubtful.

3. The presence of a fat in the diet is of no or very slight value.

4. Like many other insects, the clothes moth grows faster on flour of high than of low extraction, but even wholemeal flour is far from being an optimal diet.

5. Growth is faster at 70 % relative humidity than at 50 and 30 %, but even at 30 % most larvae complete development.

6. These findings are discussed in connexion with the natural occurrence of clothes moth larvae on woollen cloth, flour and other dried foods.

We gratefully acknowledge a grant by the Medical Research Council for the study of insect nutrition, of which this is a part.

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THE DIETETICS OF THE CATERPILLARS OF THREE *EPHESTIA* SPECIES, *E. KUEHNIELLA*, *E. ELUTELLA*, AND *E. CAUTELLA*, AND OF A CLOSELY RELATED SPECIES, *PLODIA INTERPUNCTELLA*

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(With Twelve Text-figures)

In the course of a previous analysis of the dietetics of several insects found on dried foods (Fraenkel & Blewett, 1943*a*), it was discovered that the larva of *Ephestia kuehniella*, which normally feeds on flour, grew badly, unlike the larvae of several beetles, on artificial diets consisting of casein, glucose, cholesterol and salts and the moths failed to emerge. Later the investigation was extended to *E. elutella*, which is known to infest such divergent materials as grain, tobacco, caçao beans and dried fruit. *E. elutella* grew fairly well on artificial diets, but again the moths failed to emerge from the pupae. Here the work was held up until it was discovered that *Ephestia* required, for successful growth and metamorphosis, fat-soluble factors which can be supplied with wheat-germ oil (Fraenkel & Blewett, 1945). In the present paper, which mainly deals with the requirements for carbohydrates, sterols and vitamins of the B complex, most of the diets described contain wheat-germ oil. An analysis of the factors concerned in wheat-germ oil will be given in the following paper.

Most of the experiments to be described were carried out with *E. kuehniella* and *E. elutella*, but, in addition, a number of tests were performed with another *Ephestia* species (*cautella*), and a very closely allied species, *Plodia interpunctella* which lives under very similar conditions to the *Ephestia* species.

METHODS

All the tests were performed at 25° C. and 70% relative humidity. The methods employed are similar to those already described (Fraenkel & Blewett, 1943*a*, *d*). As the caterpillars of *Ephestia* are considerably larger than the larvae of the beetles used in previous experiments, five larvae (instead of ten) were always grown together in a 2 × 1 in. tube which contained 3 g. of food (instead of 2). In view of the great number of individual tests it was not found practicable to grow each caterpillar singly. Each test was performed with twenty larvae. When the larvae were fully grown and started wandering, a strip of corrugated paper, about 2 × 1 in., was inserted into each tube. Most larvae of the three *Ephestia* species, but not of *Plodia*, pupated in the folds of the corrugated paper. By holding the papers toward the light it was possible to distinguish the darker and shorter pupa from the larva, and this method had also the additional advantage that the dates of pupation could be marked on the papers. *Plodia* pupated inside the food and not in papers, which made it impossible to observe the date of pupation.

The efficiency of a diet for the three *Ephestia* species was estimated by the length of the larval stage and the number pupating and for *Plodia* by the larval plus pupal stage and the number of moths emerging. The duration of the pupal stage is independent of the duration of the larval stage, and lasts, at 25° C., about 10–12 days with the four species in question.

The method of preparing the diets has been slightly modified since the earlier descriptions (Fraenkel & Blewett, 1943*a*, *b*, *d*). The salt mixture was not added as a suspension to each individual diet but was mixed into a large quantity of casein with some water and the mixture dried and ground. This ensured a more homogeneous distribution of salts. Another modification, introduced in the course of this investigation, consisted of regrinding the diets in a mortar after they had stood for one or more days at 25° C. and 70% R.H., and before larvae were added. This ensured a more homogeneous mixture and prevented caking.

All the curves show the total number of pupae or adults formed, plotted against time.

Most experiments to be described below are a modification of a diet which had proved adequate for a number of insects (Fraenkel & Blewett, 1943 *a*). It consisted of

Casein, vitamin and fat free (E or A/E, Glaxo Laboratories)	50 parts
Glucose (or starch)	50 parts
Dried debittered brewers' yeast (Glaxo Laboratories)	5 parts
Cholesterol	1 part
McCollum's salt mixture	2 parts
Water	10 parts
(After mixing and equilibrating at 70 % R.H. the water content of the diet is approximately 10–12 %)	
Wheat-germ oil (added after the necessity for it had been discovered)	*c.* 1 part

The ingredients of the dry diet add, therefore, up to 109 parts. For convenience we speak of percentages instead of parts, which introduces only a small error. In tests in which the amount of carbohydrates was modified, it was substituted by an equal amount of casein so that carbohydrates and casein together always constituted 100 parts. In earlier experiments 15 parts of water were added to the diets. As the dry ingredients were not water free the initial water content of the diets must have been well above 15 %, and although the diets gradually lost water while standing at 70 % R.H., on some occasions diets became mouldy. In later tests only 10 parts of water were added.

Most results depend on a relative comparison of the efficiency of different diets. The absolute results obtained on a particular diet on different occasions are not always identical, and it is therefore important that a comparison of diets be made in the same batch of larvae grown simultaneously. Most figures in this paper represent results obtained in one set of tests. An example of how widely results obtained on different occasions may vary is given in Fig. 7, which also shows that otherwise the results are virtually identical.

THE BASIC FOOD REQUIREMENTS

Ephestia elutella grows about as fast on the artificial diet described above as on weatings (one of the offal fractions obtained in the milling of white flour) which so far has proved the best natural food for *E. elutella* (cf. Figs. 1, 6). The artificial diet, supplemented by wheat-germ oil, therefore constitutes, as far as we know at present, an optimal diet. Very little or no growth takes place if glucose is omitted. Growth is very slow when the amount of glucose is reduced from 50 to 20 %. With 80 % glucose growth is noticeably faster than with 50 %, but on some occasions mortality was higher at 80 than at 50 % (Fig. 1). In nearly all the tests with *E. elutella* described in this paper diets with 50 % glucose were used. The results obtained in corresponding tests with *E. cautella* and *Plodia* were much the same (Fig. 2). Growth on an artificial diet with 50 parts glucose was about as good as on weatings. Without glucose there was no growth, and on 20 % glucose it was much slower than on 50 %. With 80 % glucose growth was always faster, but the mortality was in some cases higher.

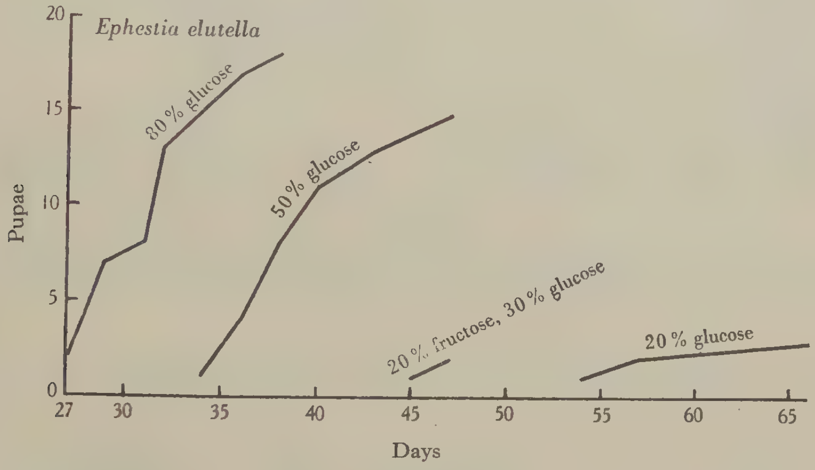

Fig. 1. Growth of *Ephestia elutella* on artificial diets containing varying amounts of glucose or fructose.

With *Ephestia kuehniella* (Fig. 3) a diet with 80 % glucose became almost as efficient as wholemeal flour, which may be considered as an optimal diet for this species. With 50 % glucose, growth was relatively much slower if compared with the other species, and hardly any growth took place with 20 % glucose. 80 % glucose was used for all tests described.

E. kuehniella grew equally well when starch (maize) was substituted for glucose and grew very much better on 50% starch than 50% glucose (Fig. 3). The other three species, strangely, either failed to grow with starch or grew exceedingly slowly. This result was unexpected as all three species are known as pests on cereals. In order to account for the inability of *E. elutella* to grow on starch a rough comparison was made of the concentration of amylase in the gut of *E. kuehniella* and *elutella*, using Cole's method of the 'first change' (Cole, 1933, Ex. 253). No difference was discovered in the rate of decoloration of a starch solution with enzyme suspensions from the guts of the two species.

E. elutella, *E. cautella* and *Plodia interpunctella* often occur as pests on dried fruit. The sugar content of dried fruit is high, and much of it consists of fructose. The water content of dried fruit is also usually higher than that of our artificial diets, or of cereals. It was therefore considered possible that adding fructose to diets might improve their efficiency, either because of intrinsic nutritional properties of fructose, or because of the increase in water content which the highly hygroscopic fructose would induce in diets. With 30% fructose and 20% glucose, growth of these three species was usually inferior to that on 50% glucose, and in all cases the mortality was high (Fig. 1). With *Ephestia kuehniella* on a diet containing 60% glucose and 20% fructose growth was slightly faster than on one with 80% glucose (Fig. 4). It

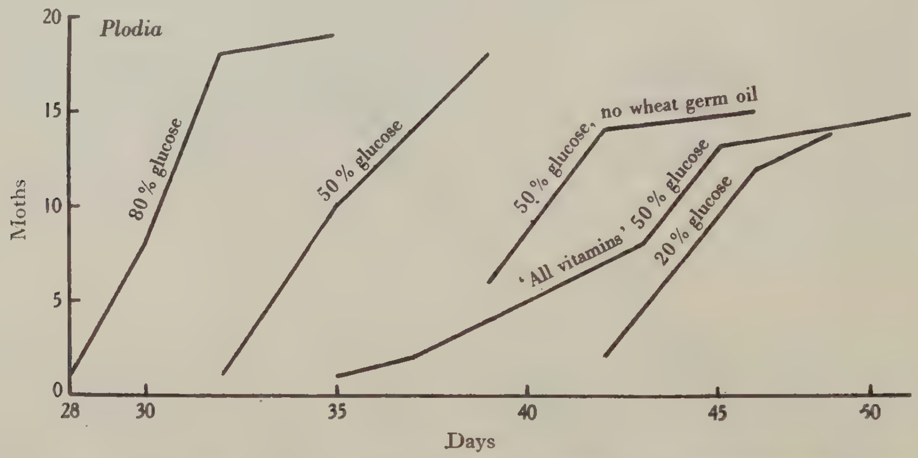

Fig. 2. Growths of *Plodia interpunctella* on artificial diets in the presence of varying amounts of glucose. All diets contain 5% yeast, except one ('all vitamins'), which contains a mixture of pure vitamins of the B complex and the water-insoluble fraction of yeast.

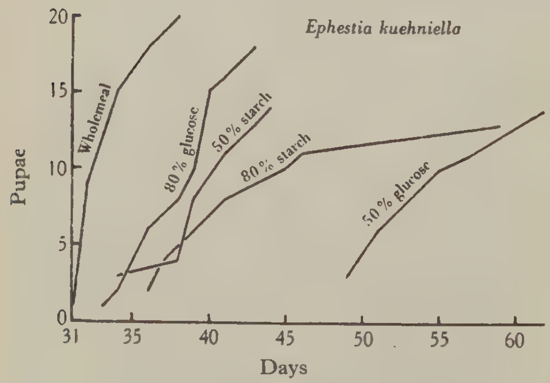

Fig. 3. Growth of *Ephestia kuehniella* in the presence of varying amounts of glucose or starch.

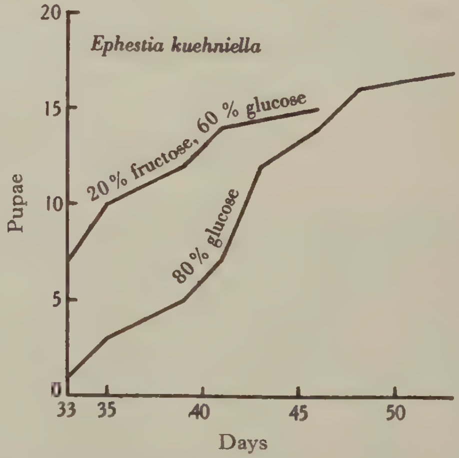

Fig. 4. Growth of *Ephestia kuehniella* in the presence of 80% glucose or 60% glucose plus 20% fructose.

appears, therefore, that the effect of fructose on the diet, for reasons not yet elucidated, was detrimental, except for *E. kuehniella*.

Testing for the importance of cholesterol in the artificial diet is complicated by the fact that a sterol deficiency can only be produced in the absence of wheat-germ oil, which is important for successful emergence of adult moths in the three *Ephestia* species, and also affects the growth rate. The need of *E. kuehniella* for sterol in the diet has previously been demonstrated by us (Fraenkel & Blewett, 1943*a*, *c*), using wholemeal flour extracted with chloroform. *E. elutella* on our basic diet, without wheat-germ oil, grew very much better in the presence of cholesterol than in its absence, and a corresponding difference in growth rate was also found when aqueous yeast extract and 'insoluble yeast' (yeast exhaustively extracted with water (Fraenkel & Blewett, 1943*a*)) was given instead of yeast (Fig. 5). The fact that some slow growth took place in the absence of cholesterol is almost certainly explained by the small amounts of ergosterol which are supplied to the diets with yeast. No growth took place on a diet which contained an aqueous yeast extract and no 'insoluble yeast' nor cholesterol, and which must have been virtually free of sterols.

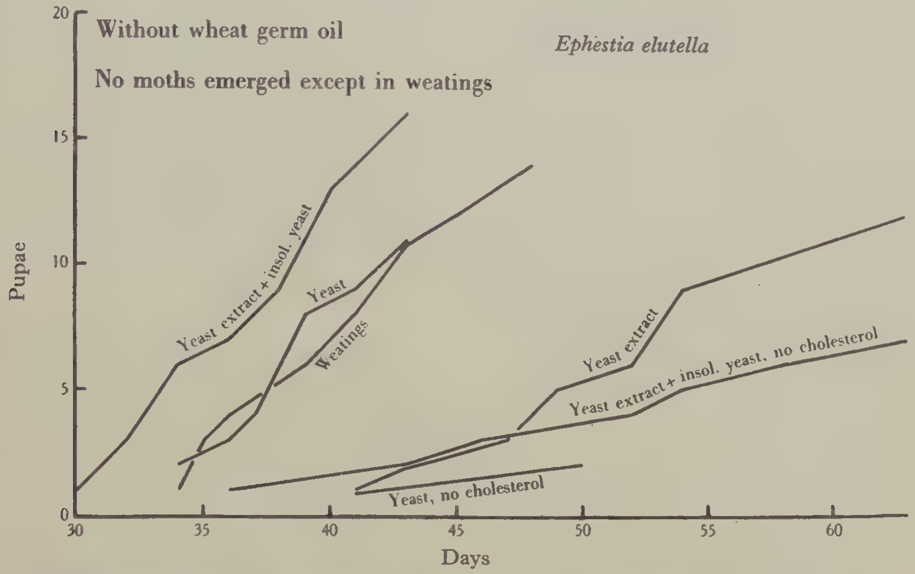

Fig. 5. Growth of *Ephestia elutella* on artificial diets with various combinations of yeast, yeast extract, insoluble yeast and cholesterol. Diets do not contain wheat-germ oil. No moths emerged except on weatings.

Table 1. *Growth of* Ephestia kuehniella, E. elutella, E. cautella *and* Plodia interpunctella *on certain artificial diets*

Diet contains	*E. kuehniella*	*E. elutella*	*E. cautella*	*Plodia*
Wholemeal flour	+++++	++	±	±
80% glucose	++++	++++	++++	++++
50% glucose	++	++++	+++	+++
20% glucose	—	+	+	++
60% glucose, 20% fructose	++++	Not done	Not done	Not done
30% glucose, 20% fructose	Not done	++?	+++?	+++?
80% starch	++++	Not done	Not done	Not done
50% starch	++++	±	—	—
No carbohydrate	—	—	—	—
No cholestrol	—	—	—	—

The results of this section are summarized in Table 1 using symbols which are self-explanatory.

REQUIREMENTS OF VITAMINS OF THE B COMPLEX

(*a*) *Ephestia elutella*

After having ascertained the importance of the proximal constituents of the artificial diet described on

p. 163, the next step consisted in analysing the factors contained in yeast. Yeast was divided into a water-soluble and a water-insoluble fraction. The water-soluble extract was prepared from fresh brewers' (pitching) yeast according to Chick & Roscoe (1930), so that 1 c.c. of the extract corresponded roughly to 1 g. of the dry yeast. 15 parts of this extract were used in the diets and no further water was given. This quantity had been found previously to be adequate for optimal growth of other insects (Fraenkel & Blewett, 1943*a*). It corresponds theoretically to 15 parts of dry yeast in the diet as against 5 parts in the original diet, but it is highly improbable that the water extract contains the whole of the water-soluble fraction of yeast. Water 'insoluble yeast' is the residue from the preparation of yeast extract which has been further treated to nine extractions with large quantities of boiling water for 1 hr. each.

In an earlier experiment (Fig. 5) where wheat-germ oil was not used growth was somewhat quicker with both yeast extract and insoluble yeast in the diet than with yeast, which may have been due to larger amounts of some soluble factor in the diet. With insoluble yeast alone there was no growth. With yeast extract alone growth became very much delayed. It was therefore clear that *E. elutella* required both soluble and insoluble factors from yeast.

In the following tests yeast extract was replaced by a mixture of the known constituents of the vitamin B complex, while 'insoluble yeast' was retained at first as a source of insoluble factors. The full diet, subsequently called 'all vitamins' diet, consisted of

	Parts		μg./g.
Casein	50	Thiamin (B_1)	50
Glucose	50	Riboflavin	50
Cholesterol	1	Nicotinic acid	50
Salt mixture	2	Pyridoxin (B_6)	50
Insoluble yeast	5	Pantothenic acid	50
Wheat-germ oil	½–1	*p*-Amino-benzoic acid	50
		Choline chloride	500
		Inositol	500

On this diet growth was relatively good, but noticeably slower than on a diet which contained whole yeast (Fig. 6). This indicated that it was lacking in some unknown factor(s). In a series of tests the importance of each factor of the B complex was then investigated by leaving vitamins out of the diet one at a time. No growth took place in the absence of thiamin, riboflavin, nicotinic acid, pyridoxin, or pantothenic acid. In the absence of choline growth proceeded at a considerably retarded rate, but most larvae ultimately pupated and emerged (Fig. 6). There was no indication that either inositol or *p*-amino-benzoic acid is of any importance for *E. elutella* (Fig. 7). Without them growth was in every respect as good as with them. It was considered possible that significant amounts of inositol might have been supplied in the diet with 'insoluble yeast', and therefore a further comparison was made of two diets which contained biotin but no 'insoluble yeast'. Here again, the presence or absence of inositol made no difference to the efficiency of the diet (Fig. 8).

Leaving out insoluble yeast from the 'all vitamins' diet had the effect of slowing down growth and increasing mortality (Fig. 8). This deficiency was partly or entirely made good by the addition of biotin. The amounts of biotin used were relatively small, 0·1 μg./g., which is the amount found to be optimal in earlier experiments with another insect, *Tribolium* (Fraenkel & Blewett, 1943*b*). In one test (Fig. 8) a diet with biotin was in every respect as good as one with insoluble yeast, while in another test (Fig. 6) the biotin diet was considerably inferior. The better effect was obtained with a sample of free biotin, while in the other test biotin methyl ester was used. It is not impossible that the difference in efficiency of the two samples was due to this difference in constitution. In earlier tests with *Tribolium* free biotin also appeared to be more efficient than the methyl ester.

(*b*) *Ephestia kuehniella*

The diet which was used for investigating the requirements of the members of the vitamin B complex was the same as that described above for *E. elutella* except that it contained 80 parts of glucose instead of 50. Here, as with *E. elutella*, a diet which contained eight known members of the B complex, and in addition insoluble yeast, was considerably inferior to a diet which contains yeast instead of a mixture of pure vitamins (Fig. 9). In the absence of either choline or inositol the diet appeared to become less efficient, and without *p*-amino-benzoic acid more efficient (Fig. 9). On these four diets, pupation started at almost exactly the same time, and it is possible that the apparent differences in efficiency are not significant. In the absence of either thiamin, nicotinic acid, pyridoxin or pantothenic acid, no growth took place. Without riboflavin, unlike the result obtained with *E. elutella*, sixteen larvae out of twenty eventually completed development. In these tests the quantities of vitamins used were arbitrary and almost certainly much in excess of the amounts required. Experiments designed with the view of determining the minimum optimal requirements were only undertaken with nicotinic acid. Graded responses were obtained to graded doses of nicotinic acid, with an optimum effect at 8 μg./g. of the diet and a threshold effect with 4 μg./g. (Fig. 10).

Even in the presence of all the known vitamins of the B complex, the larvae of *E. kuehniella*, similar to those of *E. elutella*, grow considerably more slowly than on a diet with yeast (Figs. 9, 10). This indicates that important unknown factors are still missing. With insoluble yeast left out from the diet, growth becomes very much slower still, and only eleven larvae completed development. This deficiency was entirely, or almost entirely, remedied by adding biotin (Fig. 11). Biotin, therefore, is an essential growth factor for *E. kuehniella*.

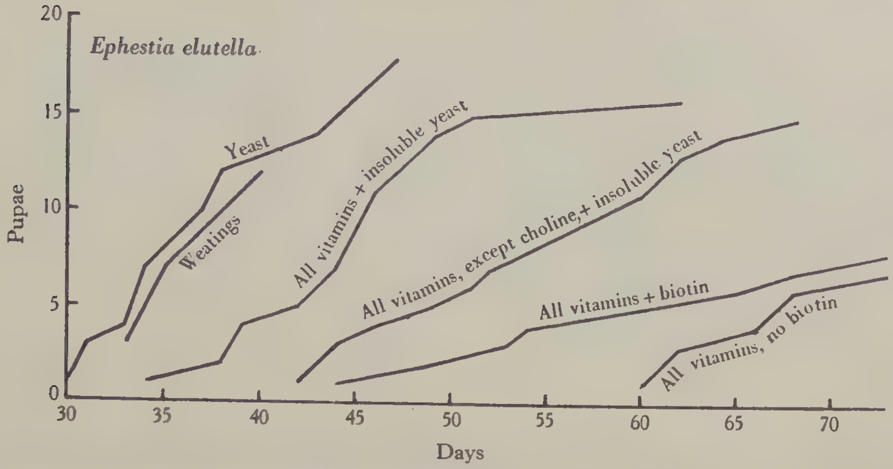

Fig. 6. Growth of *Ephestia elutella* on diets containing a mixture of the vitamins of the B complex and in the presence or absence of insoluble yeast, choline or biotin.

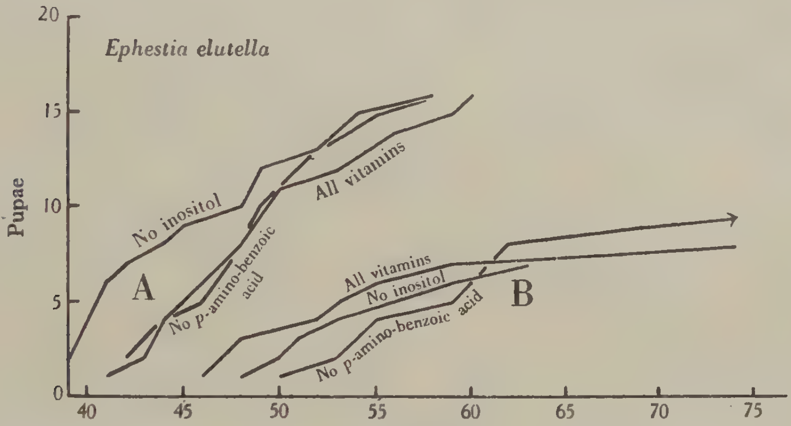

Fig. 7. Growth of *Ephestia elutella* on diets containing all the known factors of the vitamin B complex and in the presence or absence of inositol or *p*-amino-benzoic acid. A and B represent sets of tests carried out at different times.

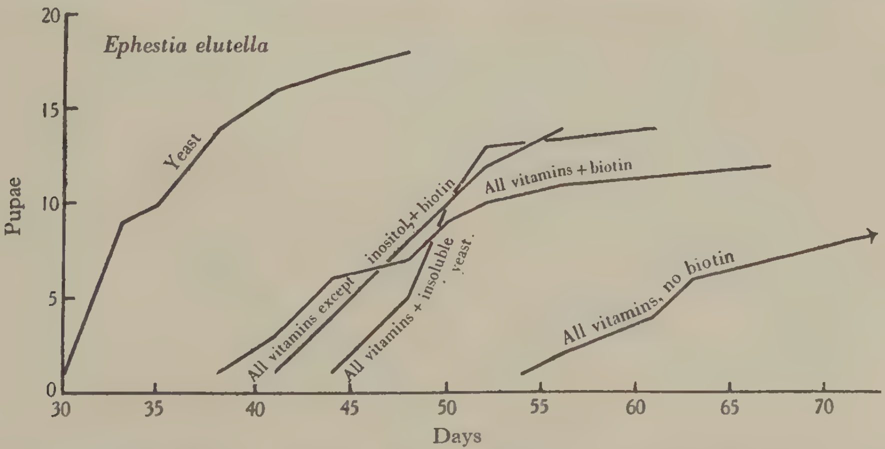

Fig. 8. Growth of *Ephestia elutella* on artificial diets in the presence or absence of insoluble yeast, biotin or inositol.

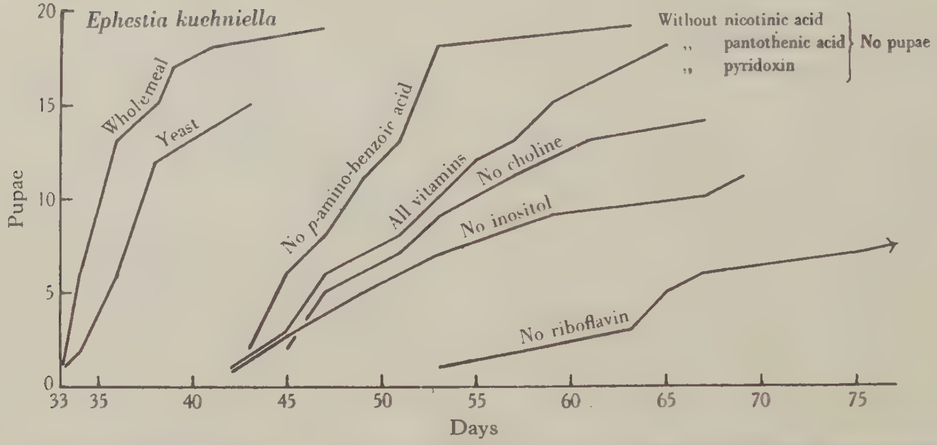

Fig. 9. Growth of *Ephestia kuehniella* on diets containing 'all vitamins' of the B complex, and on similar diets in the absence of *p*-amino-benzoic acid, choline, inositol or riboflavin. No growth took place in the absence of thiamin, nicotinic acid, pyridoxin or pantothenic acid.

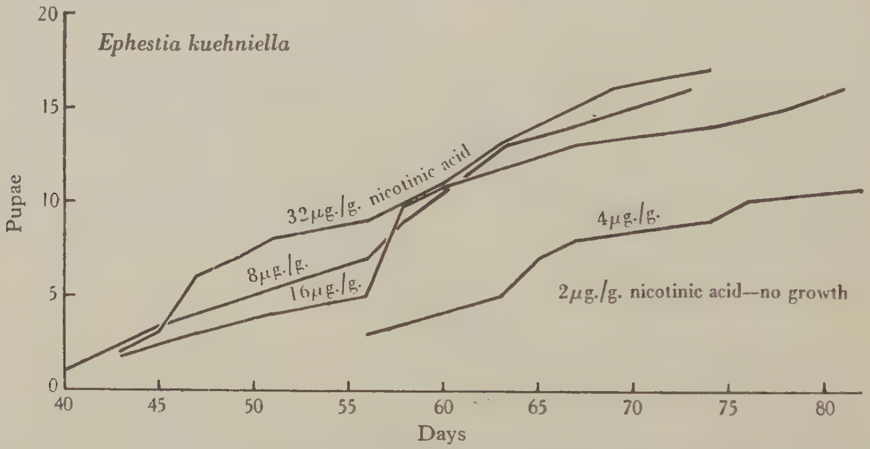

Fig. 10. Growth of *Ephestia kuehniella* on nicotinic acid-free diets, with the addition of graded doses of nicotinic acid.

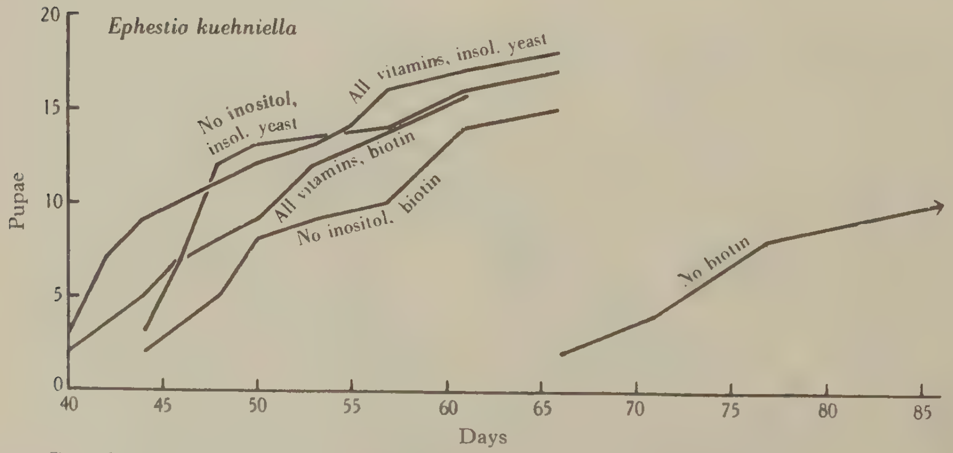

Fig. 11. Growth of *Ephestia kuehniella* on artificial diets and in the presence or absence of biotin or inositol.

In view of the uncertain result about the requirements for inositol, obtained in earlier tests (Fig. 9), another series of tests was set up, in which the effect of omitting inositol was investigated in two different diets, one containing insoluble yeast and the other biotin. In both cases there was a slight delay in growth in the diets which did not contain inositol (Fig. 11), and this, together with our earlier result, makes it very probable that inositol is of some nutritional significance for *E. kuehniella*.

(*c*) *Ephestia cautella* and *Plodia interpunctella*

On a diet containing all the known members of the vitamin B complex, and the insoluble fraction of yeast, these two species reacted very similarly to *Ephestia kuehniella* and *elutella*. Growth took place, but at a greatly reduced rate (Fig. 2). The requirements for single factors of the B complex have not yet been investigated, except for a test with biotin on *Plodia*. Here the result was very similar to that with *Ephestia kuehniella*. In the absence of biotin, hardly any growth took place, and only one out of twenty completed development while in the presence of biotin growth was slow and seven larvae completed development.

DISCUSSION

The present results fully explain why on a former occasion (Fraenkel & Blewett, 1943 *a*) we did not succeed in growing *Ephestia kuehniella* on an artificial diet with glucose as the carbohydrate. This failure was mainly due to the absence of wheat-germ oil and partly to a suboptimal concentration of glucose (50 instead of 80 %).

The two *Ephestia* species *kuehniella* and *elutella*, in their requirements for vitamins of the B complex, closely resemble several other insects investigated by us which do not obtain additional vitamins from intracellular symbionts, viz. *Tribolium confusum*, *Ptinus tectus* (Fraenkel & Blewett, 1943 *b*), *Tenebrio molitor* and *Dermestes vulpinus* (unpublished). The five important members of the vitamin B group, thiamin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid, are necessary for all these insects. *p*-Aminobenzoic acid is certainly not required and its inclusion is sometimes detrimental. With regard to inositol, we have now obtained evidence that it is of some nutritional significance for *Ephestia kuehniella*, an evidence which so far has been lacking for other insects. Choline chloride is required for *E. elutella*, *Ptinus*, *Dermestes* and *Tenebrio*, but possibly not for *Ephestia kuehniella* and *Tribolium*. *Ephestia kuehniella* and *Plodia interpunctella*, and, to a lesser degree, also *E. elutella* and *Tribolium* require biotin. On a diet which contains all the *known* members of the vitamin B complex, the three *Ephestia* species and *Plodia* grow relatively well, but the growth rate is far from being optimal. This indicates that other unknown components of the vitamin B complex are missing, though they are of lesser importance than the five known factors mentioned above.

An interesting difference between *Ephestia kuehniella* and *elutella* concerns the importance of riboflavin in the diet. *E. elutella* entirely fails to grow in the absence of riboflavin, while *E. kuehniella* grows satisfactorily, though at a rather slow rate. This perhaps partly explains why *E. kuehniella* grows so much better on flour of a low extraction than *E. elutella*. Grain is a more inferior source of riboflavin than of most of the other known factors of the B complex. Patent flour contains only very minute quantities of riboflavin (Table 2), and we have demonstrated that lack of riboflavin is the chief reason for the slow growth of *Tribolium confusum* on it (Fraenkel & Blewett, 1941). *Ephestia kuehniella* also grows very slowly on patent flour (Fraenkel & Blewett, 1943 *d*), but the essential feature is that it grows at all. It is suggested here that *E. kuehniella* can grow on patent flour by virtue of being relatively insensitive to the lack of riboflavin, and this would account for its widespread occurrence in flour mills.

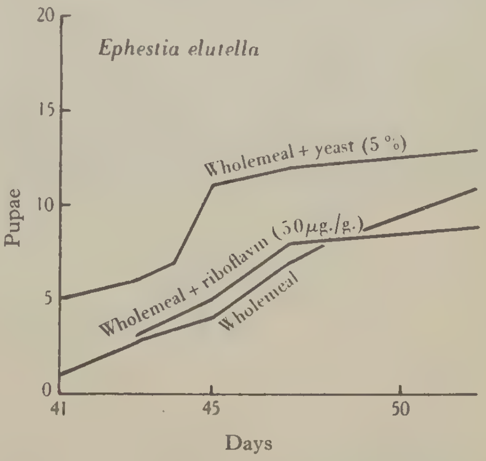

Fig. 12. Growth of *Ephestia elutella* on wholemeal flour, and with the addition of yeast or riboflavin.

E. elutella, by way of contrast, does not grow on low-extraction flour. Even on wholemeal flour growth is relatively slow. It is better on middlings, a milling fraction rich in B vitamins, and also noticeably better on wholemeal flour to which 5 % yeast has been added (Fig. 12). This suggests that a limiting factor for *E. elutella* on wholemeal flour is a lack of some vitamins of the B group. It was thought that this may perhaps be due to a shortage of riboflavin in wholemeal flour. Addition of riboflavin, however, did not improve growth on wholemeal flour (Fig. 12).

There is, however, another factor which limits the growth of *E. elutella*, *E. cautella* and *Plodia* on flour. These three species, which grew very well on an artificial diet containing glucose, unexpectedly grew hardly at all on the same diet where glucose was replaced by starch. *Ephestia kuehniella*. on the other hand, grows about equally well on starch or glucose. This result was somewhat surprising, because all four species are known as pests on grain and cereal products. According to several authorities (e.g. Cotton & Good, 1937), *Plodia interpunctella* is a major pest and *Ephestia cautella* and *elutella* are minor pests on grain and milled cereal products of all kinds. In feeding tests with *Plodia* and *Ephestia cautella* on flours of different extraction the results were similar to those previously reported for *E. elutella* (Fraenkel & Blewett, 1943 *d*). There was no growth on the low-extraction flours (40 and 72 %), but even on wholemeal flour hardly any growth took place. On whole grain, *Plodia* and *Ephestia cautella*, in our experiments, also grew slowly and only very few specimens completed development. Like *E. elutella* these two species feed exclusively on the embryo, leaving the endosperm untouched, while *E. kuehniella* feeds on both embryo and endosperm. It is generally believed that pure wheat germ (as distinct from commercial germ, which as a milling product is always contaminated with endosperm) contains no starch. The sugar content of germ has been given as about 10 % (Booth, Carter, Jones & Moran, 1941), but there is reason to believe that this is an underestimate. (Whole wheat contains only ½–1 % sugar.) It therefore seems feasible that on high-extraction milling fractions (such as middlings, weatings, germ, etc.) *E. elutella*, *E. cautella* and *Plodia* may successfully feed by picking out such particles of germ or perhaps bran which are low in starch and high in sugar and vitamins of the B complex. It is interesting to note that the effect of wholemeal flour on our four species is very much what we would expect from the result on artificial diets. For *Ephestia kuehniella*, which grows very well on starch diets, wholemeal flour is an optimal diet. *E. elutella*, which grows very slowly on starch diets, also grows very slowly on wholemeal flour though most larvae ultimately pupate. *E. cautella* and *Plodia* fail to grow on either diet (Table 1).

Ephestia elutella is also known as a pest on such divergent substances as cacao beans, dried fruit, nuts and tobacco, and as this species does not grow in the absence of most of the vitamins of the B complex it must be assumed that these foods contain sufficient amounts of them. Growth on most of these substances is slow, and we would not expect them to contain necessarily optimal amounts of all vitamins. Figures for thiamin, riboflavin and nicotinic acid of some of these foods have only very recently become known, and a comparison with corresponding figures for wholemeal flour shows (Table 2) that the requirements of *E. elutella* of some B vitamins must be very low. Nothing is known about vitamins in cured tobacco, but, judging from the food requirements of *E. elutella*, it must be assumed to contain substantial quantities of carbohydrates and of vitamins of the B complex.

Table 2. *Composition of certain dried foods*

	Carbo-hydrates %	Thiamin mg./100 g.	Riboflavin mg./100 g.	Nicotinic acid mg./100 g.	Source
Cocoa	31	0·09	0·45	1·5	N.R.C. (1944)
Ground nuts	24	0·4	0·13	16	N.R.C.
Dried prunes	71	0·09	0·1	1·5	N.R.C.
Raisins	71	0·14	0·1	0·5	N.R.C.
Wholemeal flour	72	0·47	0·11	4·1	N.R.C.
Patent flour	75	0·07	0·03	0·6	Cheldelin & Williams (1942)

SUMMARY

1. The larvae of four species of moths, *Ephestia kuehniella*, *E. elutella*, *E. cautella* and *Plodia interpunctella*, have been grown successfully on artificial diets, consisting of casein, glucose, yeast, cholesterol, a salt mixture, wheat-germ oil and water.

2. The requirements for carbohydrates are high. Growth of *Ephestia kuehniella* is optimal with 80 % glucose in the diet. The other three species also grow fastest with 80 % glucose, but survival is usually better with 50 % glucose. With smaller quantities of glucose growth becomes slower, and little or no growth takes place with 20 % glucose or less.

3. Cholesterol is an essential constituent of the diet for all four species.

4. On diets which contain the known factors of the vitamin B complex in pure substance in place of yeast, all four species grow relatively well, but the growth rate becomes noticeably slower. This indicates the presence of further important growth factors in yeast.

5. Of the known factors of the vitamin B complex, the following are indispensable for *E. kuehniella* and *elutella*: thiamin, nicotinic acid, pyridoxin and pantothenic acid. Riboflavin is also indispensable for *E. elutella*, while in its absence *E. kuehniella* grows very slowly. Choline chloride greatly improves growth of *E. elutella*, inositol has some beneficial effect on that of *E. kuehniella*, and *p*-amino-benzoic acid does not seem to be necessary for either species. Biotin is an

important growth factor for *E. kuehniella* and *Plodia*, and, to a somewhat lesser degree, also improves growth of *Ephestia elutella*.

6. Diets in which glucose is replaced by starch are excellent for *E. kuehniella*, but unsuitable for the other three species.
7. These findings are discussed in connexion with the natural occurrence and the type of feeding of the four species on flour, whole grain and other dried foods.

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LINOLEIC ACID, VITAMIN E AND OTHER FAT-SOLUBLE SUBSTANCES IN THE NUTRITION OF CERTAIN INSECTS, *EPHESTIA KUEHNIELLA*, *E. ELUTELLA*, *E. CAUTELLA* AND *PLODIA INTERPUNCTELLA* (LEP.)

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(*Received* 14 *November* 1945)

(With Sixteen Text-figures)

In a previous publication (Fraenkel & Blewett, 1943) it has been shown that a number of insects which normally live on wholemeal flour or other cereal products grow badly or not at all on flour thoroughly extracted with chloroform unless a sterol, e.g. cholesterol, is added to the diet. For several beetle larvae the diet becomes optimal upon addition of a pure sterol, but for the caterpillars of a moth, *Ephestia kuehniella*, wheat-germ oil has a much better effect than a sterol. Using artificial diets, consisting of glucose, casein, yeast and cholesterol, it was found that *E. kuehniella* grew well in the presence of wheat-germ oil and badly without it. Subsequently it emerged (Fraenkel & Blewett, 1946*b*) that the closely related species *E. elutella* grew fairly well on similar diets in the absence of wheat-germ oil and pupated normally, but the adults invariably failed to emerge from the pupae. With wheat-germ oil, growth was fast and emergence normal. Analysis of the factors in wheat-germ oil which are required by the moths of the genus *Ephestia* is the subject of the present paper. Some of the results have already been shortly reported (Fraenkel & Blewett, 1945).

METHODS

The nutritional requirements of the moths in question are fully described in a previous publication (Fraenkel & Blewett, 1946*b*) which also contains full details about the methods of computing the diets, breeding and testing. The basal diet used in all tests with *E. kuehniella* consisted of

Casein (A/E, E or 'extracted', Glaxo Lab.)	20 parts
Glucose (anhydr.)	80 parts
Yeast (dried brewers', debittered, Glaxo Lab.)	5 parts
Cholesterol	1 part
McCollum's Salt Mixture no. 185	2 parts
Water	10 parts

The diet used for *E. elutella*, *E. cautella* and *Plodia interpunctella* was the same, except that it contained 50 parts of casein and 50 parts of glucose.

The quantity of wheat-germ oil in the diets was usually of the order of 1 %, or slightly less, of the dry diet, except where otherwise stated. The saponifiable fraction of wheat-germ oil was supplied in the same quantities, and the unsaponifiable fraction was about ½ % of the dry diet. For the saponification we followed the instructions of Hilditch (1940), with only slight modifications. 20 g. of oil were saponified for 3 hr. in a solution of 5 g. KOH in 100 g. 95 % alcohol, the soaps extracted for 24 hr. with ether in continuous extractor, acidified, and the fatty acids shaken out with ether. The 'unsaponifiables' were submitted to a second saponification for 6 hr. and collected by continuous extraction with ether.

All quantities are expressed per g. of the dry diet, except where otherwise stated. As in previous publications, many of the results are graphically summarized in curves in which the total number of pupae formed is plotted against time. Each figure represents results obtained in one set of tests.

EXPERIMENTS

A. *Requirements of wheat-germ oil*

If the artificial diet described above also contains wheat-germ oil, *Ephestia kuehniella* grows almost as fast on it as on wholemeal flour. In the absence of wheat-germ oil growth is very slow and the mortality often high. In the tests recorded in Fig. 5, for instance, only three out of twenty larvae pupated after a long delay and all the moths failed to emerge from the pupal cases. With *E. elutella* the result is slightly different. In the absence of wheat-germ oil growth is also impaired but sometimes to a lesser degree, and most larvae eventually pupate. Again, moths never emerge from these pupae (Fig. 6 and Fraenkel & Blewett, 1946*b*, Fig. 5).

An examination of old pupae which failed to emerge invariably showed the presence of fully developed moths inside the pupal cases; they are pigmented and in the state in which they would normally emerge. They are alive if examined on the 12th or 13th day after pupation, the normal duration of the pupal stage at 25° C., but die within 1 or 2 days. In some cases there were signs of an attempted emergence, such as a burst head capsule and the freeing of parts of the head, the proboscis or the anterior legs, and occasionally a moth emerged in a malformed state. With insufficient wheat-germ oil moths emerge with wings partly or entirely lacking in scales. In a series of diets with graded doses of wheat-germ oil all stages between failure to emerge and normal moths are produced. With *E. kuehniella* the moths are normal with 4·3 mg. wheat-germ oil/g. of the diet. Lowering the amount of wheat-germ oil has the effect of producing moths with increasingly naked wings. For the purpose of classifying degrees of deficiency we are distinguishing the following eight classes, illustrated in Fig. 1:

Class	Forewings	Hindwings
1	Normal	Normal
2	Normal	Scales missing, or partly missing from lower half
3	Some scales missing	No scales, except on frontal rim
4	Bare circular patch	No scales except in narrow frontal zone
5	Distal part bare	No scales
6	No scales, except in basal zone	No scales
7	No scales on wings, wings not expanded and moths often otherwise malformed	
8	No emergence	

The effects of varying amounts of wheat-germ oil in the diet of *E. kuehniella* are shown in Table 1 (1 *a–e*).

The question here arises as to the fate of the missing scales. These are found stuck on to the inside of the empty pupal cases where they are seen as dark regions after the moth has emerged. It appears that the formation of scales proceeds normally up to the time of emergence of the moth. Then during emergence, instead of separating from the pupal case and remaining on the cuticle of the adult, they separate from the adult cuticle and stick to the pupal case. As long as this occurs on parts of the wings only moths emerge normally but for the absence of scales on the wings. In the complete absence of wheat-germ oil the scales also remain stuck in other regions, especially over the abdomen, and thus emergence is made impossible. A very similar if not perhaps identical phenomenon has been described before as a gene mutation in the same species (Kühn & Henke, 1929), and we shall suggest below a possible explanation of this phenomenon.

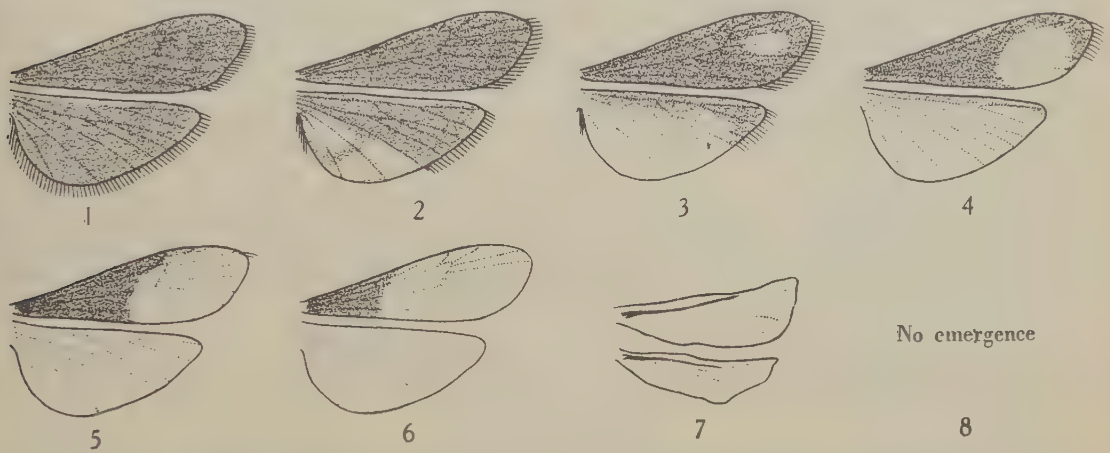

Fig. 1. Wings of *Ephestia kuehniella* (semi-diagrammatic). Classification of symptoms of linoleic acid deficiency Explanation in text.

In this paper we shall use terms such as *scale* deficiency, scale condition or scale factor when referring to a nutritional deficiency which affects the scales or emergence, as distinct from a growth deficiency whereby the rate of growth is affected. Both types of deficiency occur in *Ephestia* in connexion with fat-soluble substances in the diet. In *E. kuehniella*, growth is greatly retarded in the absence of wheat-germ oil, and increasing amounts of it have a similar improving effect on growth and scales. Fig. 2 shows the rate of growth of larvae, grown in the presence of varying amounts of wheat-germ oil, which have been analysed in Table 1 for the effect on scales and emergence. With 8·7 and 4·3 mg. of wheat-germ oil the rate of growth is optimal and the moths are normal. With decreasing amounts of wheat-germ oil rate of growth and condition of scales becomes increasingly worse, and both are at the minimum in the absence of wheat-germ oil. There was, however, an exception in the diet with 1·2 mg. wheat-germ oil where growth was very fast but the moth abnormal. In diets with 1·2 mg. and less the oil had been diluted with petroleum ether (tech., B.D.H.) and it was considered not impossible that the ether, or impurities in it, may have had a favourable effect on growth. Fast growth and good scales, therefore, do not necessarily always go together.

Table 1. *The effect of various oils and fractions of oils on the degree of linoleic acid deficiency in* Ephestia kuehniella

Additions to diets (per g.)	Classes of deficiencies (p. 173)									
	1	2	3	4	5	6	7	8	Lost	Total
1. (*a*) 8·7 mg. wheat-germ oil	17	—	—	—	—	—	—	—	—	17
(*b*) 4·3 mg. wheat-germ oil	16	—	—	—	—	—	1	—	—	17
(*c*) 2·3 mg. wheat-germ oil	5	5	1	—	—	—	—	1	5	17
(*d*) 1·2 mg. wheat-germ oil	1	5	6	1	—	—	—	—	4	17
(*e*) 0·5 mg. wheat-germ oil	—	2	2	2	4	2	2	—	—	14
2. Unsaponifiable fraction of wheat-germ oil plus:										
(*a*) 6·0 mg. linoleic acid	12	—	—	—	—	—	—	—	—	12
(*b*) 4·0 mg. linoleic acid	7	4	—	—	—	—	—	—	—	11
(*c*) 2·0 mg. linoleic acid	1	6	2	3	1	1	1	—	—	15
(*d*) 1·3 mg. linoleic acid	—	—	4	3	2	—	2	2	—	13
(*e*) 0·3 mg. linoleic acid	—	—	1	—	4	4	5	1	—	15
3. 4·0 mg. linoleic acid	3	1	1	—	3	1	4	2	1	16
4. 0·33 mg. vitamin E (Roche) plus:										
(*a*) 4·0 mg. linoleic acid	—	—	3	2	7	1	1	1	1	16
(*b*) 1·7 mg. linoleic acid	—	—	1	—	1	4	4	—	—	10
5. 0·33 mg. vitamin E (Glaxo) plus 4·0 mg. linoleic acid	8	2	1	1	—	—	—	—	3	15
6. 5 mg. unsaponifiable fraction of wheat-germ oil	—	—	—	—	—	—	5	10	—	15
7. No addition	—	—	—	—	—	—	2	11	—	13
8. Unsaponifiable fraction of wheat-germ oil plus:										
(*a*) 9·3 mg. linolenic acid	4	2	1	1	—	—	—	—	—	8
(*b*) 2·3 mg. linolenic acid	18	—	—	—	—	—	—	—	—	18
9. (*a*) 9·3 mg. linolenic acid	—	—	—	—	—	—	—	—	—	None
(*b*) 2·3 mg. linolenic acid	—	1	2	1	1	1	2	3	—	11
10. (*a*) 9·0 mg. cod-liver oil	—	—	—	—	—	—	1	10	—	11
(*b*) 3·0 mg. cod-liver oil	—	—	—	—	—	—	3	9	—	12
11. (*a*) 10·0 mg. halibut-liver oil	—	—	—	—	—	—	7	10	—	17
(*b*) 2·5 mg. halibut-liver oil	—	—	—	—	1	1	6	10	—	18
12. (*a*) 10·0 mg. butter fat	—	—	1	2	4	2	6	—	—	15
(*b*) 2·5 mg. butter fat	—	—	—	2	1	2	5	9	—	19
13. (*a*) 10 mg. lard	1?	1?	←—11—→			2?	1	—	—	16
(*b*) 10 mg. saponifiable fraction of lard	—	←—7—→			2?	2	3	1	—	15

A third effect, arising from the absence of wheat-germ oil, is increased mortality of the larva (as distinct from mortality in the pupa at the time of emergence). The number of larvae ultimately pupating is always lower in the absence of wheat-germ oil, but differs widely on different occasions. In the tests of Fig. 2, eleven out of twenty pupated, in Figs. 9 and 5 the numbers were nine and three respectively.

E. elutella in the absence of wheat-germ oil never emerges from the pupa, and there are no signs of unsuccessful attempts at emergence (Table 2 (5)). Without the oil, growth is always delayed, but, as a rule, both growth rate and mortality are less affected than with *E. kuehniella*. No test has yet been performed with *E. elutella* with graded doses of wheat-germ oil, but from tests with graded doses of linoleic acid, to be reported below, there can be no doubt that essentially the same response would have been obtained as with *E. kuehniella*.

A third representative of the genus *Ephestia*, *E. cautella*, reacts very much in the same way in the absence of wheat-germ oil: growth is delayed and the moths fail to emerge from the pupal cases.

A fourth species investigated was *Plodia interpunctella* which is the species most closely related to *Ephestia*. *Plodia* never failed to emerge in a perfect state in the absence of wheat-germ oil. Growth was retarded, but the extent of this delay was different on

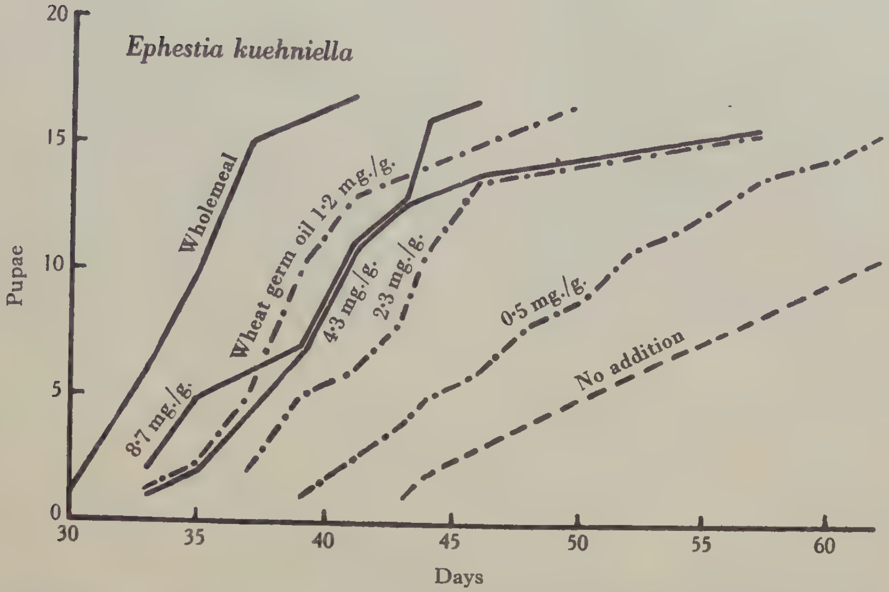

Fig. 2. Growth of *Ephestia kuehniella* on artificial diets and in the presence of graded doses of wheat-germ oil. ——Moths with perfect wings; –·–·–·– moths with wings partly or entirely lacking in scales; – – – no moths emerge. The same explanation applies to the lines of all subsequent figures.

Table 2. *The effect of different quantities of linoleic acid on the degree of linoleic acid deficiency in* Ephestia elutella

Additions to diets (per g.)	Classes of deficiencies (p. 173)									
	1	2	3	4	5	6	7	8	Lost	Total
1. 8·7 mg. wheat-germ oil	14	—	—	—	—	—	—	—	—	14
2. 5 mg. unsaponifiable fraction of wheat-germ oil plus:										
(*a*) 6 mg. linoleic acid	11	1	—	—	—	—	—	—	—	12
(*b*) 4 mg. linoleic acid	6	2	—	—	—	—	1	—	—	9
(*c*) 2 mg. linoleic acid	4	3	2	—	—	—	—	—	2	11
(*d*) 1·3 mg. linoleic acid	1	4	—	—	—	—	—	2	—	7
(*e*) 0·33 mg. linoleic acid	—	—	—	—	—	2	1	8	—	11
3. 4 mg. linoleic acid	—	—	—	—	—	—	—	4	—	4
4. 5 mg. unsaponifiable fraction of wheat-germ oil	—	—	—	—	—	—	—	7	—	7
5. No addition	—	—	—	—	—	—	—	13	—	13
6. 0·33 mg. vitamin E plus:										
(*a*) 20 mg. linoleic acid	6	—	—	—	—	—	—	1	—	7
(*b*) 10 mg. linoleic acid	7	—	—	—	—	—	—	—	—	7
(*c*) 3·3 mg. linoleic acid	6	—	—	—	—	—	—	—	—	6
(*d*) 1 mg. linoleic acid	1	←——6——→				2	—	1	—	10
(*e*) 0·33 mg. linoleic acid	—	—	—	1?	—	—	1	5	—	7

different occasions. It was at first thought that perhaps sufficient of the fat-soluble factors might have been supplied by the fat which is contained in 5 % yeast. Tests were consequently carried out with diets containing yeast which had been extracted with ether (Soxhlet). Here growth was slower than with normal yeast, but the moths always emerged normally.

B. *The saponifiable and unsaponifiable fractions of wheat-germ oil*

Wheat-germ oil was subsequently saponified, and both the resulting fractions, saponifiable and unsaponifiable, were tested separately and combined (Fig. 3). With both fractions, growth is usually, but not always, rather slower than with wheat-germ oil, which indicates changes in the resulting fractions. With the saponifiable fraction alone, the moths of both *kuehniella* and *elutella* always emerge normally, but growth is usually (*elutella*) or always (*kuehniella*) slower than with both fractions. With *kuehniella* on certain occasions (Fig. 3), but not always (Figs. 9, 14), the saponifiable fraction did not accelerate growth. With *elutella* growth is relatively less impaired in the absence of the saponifiable fraction.

With the unsaponifiable fraction alone, the moths of both species fail to emerge, but growth is always considerably faster with it than without it. The extent to which the growth rate is maintained again varies considerably on different occasions. In most experiments with *E. kuehniella* growth was slower with the saponifiable than with the unsaponifiable fraction alone, and fastest with both together (Fig. 3). On one occasion, however (Fig. 14), the saponifiable fraction was faster than the unsaponifiable fraction, but again growth was greatly superior in the presence of both. The growth-promoting effect of the unsaponifiable fraction was more marked with *kuehniella* than *elutella*. This is what one would have expected, as *elutella* grows relatively faster than *kuehniella* in the absence of wheat-germ oil. This difference in growth of these two species on a diet without wheat-germ oil is almost certainly due to the effect of yeast oil on *elutella*. On a fat-free diet, containing yeast which had been thoroughly extracted with ether (Soxhlet), *E. elutella* grew very much more slowly than with ordinary yeast, and growth was greatly improved by the addition of the unsaponifiable fraction of wheat-germ oil. It is, therefore, clear that both species require growth-promoting factors which may be supplied with the unsaponifiable fraction of wheat-germ oil.

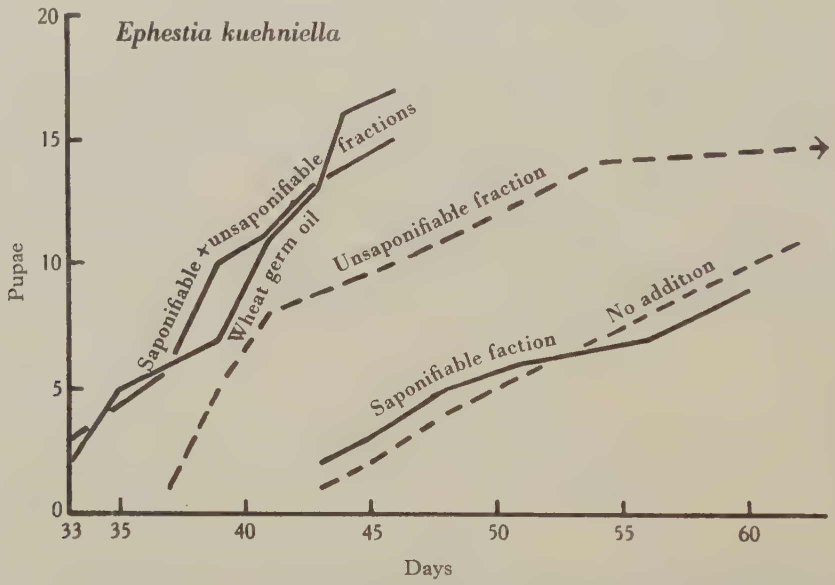

Fig. 3. Growth of *Ephestia kuehniella* on artificial diets with the addition of wheat-germ oil, or the products of saponification of wheat-germ oil.

E. cautella reacted very similarly to the other two species. With both fractions of wheat-germ oil present, normal moths emerged, and the moths were also normal with the saponifiable fraction alone. No moths emerged with the unsaponifiable fraction alone. Each fraction separately had a favourable effect on growth. Growth was delayed if the diet contained ether-extracted yeast.

It is therefore clear that for the three *Ephestia* species, emergence and good scales are bound up with a substance present in the saponifiable fraction of wheat-germ oil, while the unsaponifiable fraction contains something which improves growth.

C. *The saponifiable factor*

Wheat-germ oil contains 50–60 % of linoleic acid. A sample (from Vitamins Ltd.) which we used in the latter part of our experiments was analysed by Prof. Hilditch and found to contain 54·9 %. As linoleic acid is one of the essential fatty acids in the nutrition of the rat it was an obvious step to test its effect on *Ephestia*. In early experiments with a technical sample of linoleic acid (B.D.H.) normal moths emerged but growth remained slow. Later, pure samples were obtained by courtesy of the late Mrs Smedley-Maclean (methyl-linoleate) and Prof. G. O. Burr (ethyl-linoleate), and the results obtained with the technically impure sample were fully confirmed.

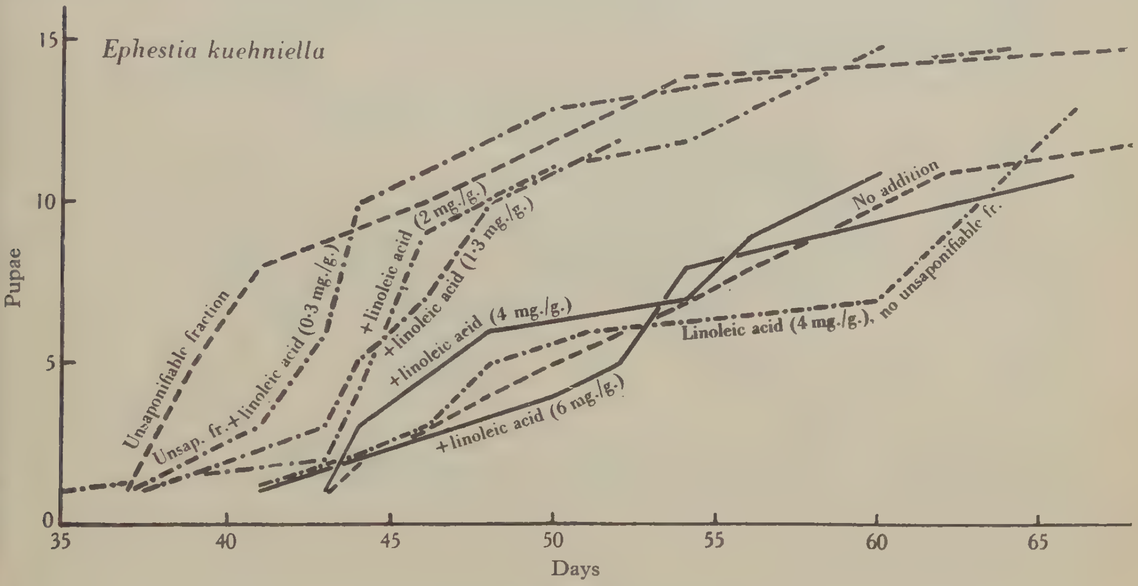

Fig. 4. Growth of *Ephestia kuehniella* on artificial diets in the presence of the unsaponifiable fraction of wheat-germ oil and of graded doses of linoleic acid.

On *E. kuehniella*, addition of linoleic acid alone to the diet hardly improved the scale condition or growth, while with *E. elutella*, on occasions, perfectly formed moths were obtained. Tests with *E. kuehniella* on diets containing the unsaponifiable fraction of wheat-germ oil in addition to pure linoleic acid were at first unsuccessful. In those earlier experiments the unsaponifiable fraction had been prepared from a sample of wheat-germ oil kept for several years in the laboratory, which might have undergone changes. Subsequently, tests carried out with the unsaponifiable fraction of a fresh sample of wheat-germ oil were completely successful. The condition of the scales and emergence now became a function of the amounts of linoleic acid in the diet, exactly as had been the case in tests with graded doses of wheat-germ oil (Table 1 (2 *a*–*e*)). With 6 mg. of linoleic acid, perfectly formed moths emerged. With 4 mg. the first moths were also perfect specimens, but later four moths emerged with slightly defective wings. With the same quantity of linoleic acid, but without the unsaponifiable fraction, the condition of the wings was very much worse (Table 1 (3)). With 2 and 1·3 mg. of linoleic acid the wings became progressively more naked, and with 0·33 mg. the majority of the wings had no scales at all and many moths were badly formed. But even such a small quantity of linoleic acid had a very marked effect on emergence, as with no linoleic acid most moths failed to emerge and all that emerged were badly formed (Table 1 (7)).

While, therefore, linoleic acid was undoubtedly the factor necessary for perfect condition of the scales, it had in this experiment, paradoxically, an adverse effect on growth (Fig. 4). With increasing quantities, as the scale condition improved, the growth rate decreased, being fastest in the diet without linoleic acid when most moths failed to emerge, and slowest with the largest quantities of linoleic acid when the moths were normal. No such adverse effect of larger doses occurred in diets with graded doses of wheat-germ oil (Fig. 2). It is therefore obvious that the adverse effects must be attributed to a deterioration in the pure linoleic acid. This also follows from a comparison of the effects of identical amounts of linoleic acid in diets containing wheat-germ oil and diets with pure linoleic acid. As already stated above the wheat-germ oil which we used contained 55 % of linoleic acid. A perusal of the data in Table 1 makes it clear that identical quantities of linoleic acid were less efficient in the pure state than when supplied in wheat-germ oil. For instance, 2·4 mg. of linoleic acid contained in 4·3 mg. wheat-germ oil were sufficient, while even 4 mg. were insufficient when supplied in the pure state in the presence of the unsaponifiable fraction, and still more so when supplied alone (Table 1 (1 *b*, 2 *b* and 3)). From tests to be described below there can be little doubt that the deterioration of linoleic acid is due to a rancidative oxidation which can be checked by adding vitamin E or other anti-oxidants.

Of other fatty acids tested, linolenic acid had a similar effect to linoleic acid. In the presence of the unsaponifiable fraction, and with 2·3 mg. linolenic acid the moths emerged perfectly and the growth rate was much better than with the unsaponifiable fraction alone (Fig. 5 and Table 1 (8)). This result was greatly superior to that obtained with a similar quantity of linoleic acid, both as regards scale condition and growth rate. But when comparing the effect with that of linoleic acid as a constituent of wheat-germ oil (Table 1) the result is about the same. Increasing the quantity of linolenic acid four times had an adverse effect on growth, obviously due to deterioration, but unexpectedly the scale condition also grew worse. Without the unsaponifiable fraction, linolenic acid deteriorated quickly. With 2·3 mg. only defective moths emerged, and none developed with the larger quantity (Table 1 (9)). In the presence of the saponifiable fraction of wheat-germ oil 2·3 mg. of linoleic acid did not improve growth and 9·2 mg. had a very detrimental effect.

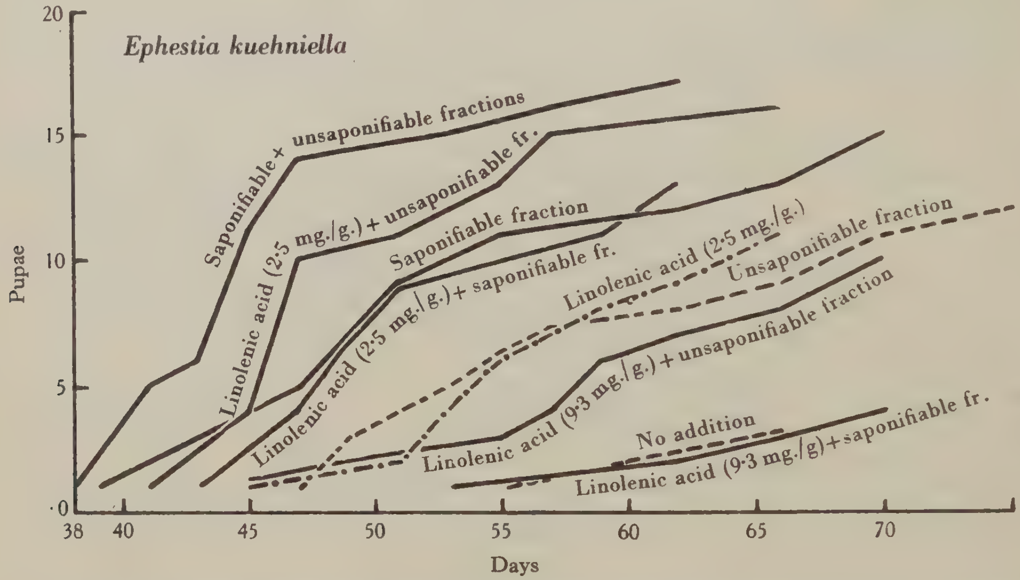

Fig. 5. Growth of *Ephestia kuehniella* on artificial diets in the presence of linolenic acid and the saponifiable or unsaponifiable fraction of wheat-germ oil.

We have so far not succeeded in procuring a good sample of arachidonic acid. An old discoloured specimen which had been kept for several years in an unsealed tube, and which was probably no longer in good condition, had no effect on scale condition. Oleic acid, of which a pure sample was provided by Dr G. King, had no effect whatsoever on scale condition but possibly a very slight positive effect on growth. Elaidic acid (also from Dr King) had no effect on scale condition, but affected the growth rate adversely. This latter may have been due to the high melting-point of this acid. The results of tests with the highly unsaturated C_{20} and C_{22} fatty acids from cod-liver oil will be described below.

With *E. elutella* a successful correction of the scale deficiency in the absence of the unsaponifiable factor was obtained at first with a technical sample of linoleic acid, and on one occasion only with pure linoleic acid. In other experiments linoleic acid alone never brought about emergence of the adult moth, but the scale-protecting effect of linoleic acid was fully operative in diets which contained in addition either the unsaponifiable fraction of wheat-germ oil, or vitamin E. A series of tests with graded doses of linoleic acid, and with the unsaponifiable fraction of wheat-germ oil, gave a graded response with regard to scale condition very similar to that obtained in the corresponding test with *E. kuehniella* (Table 2 (2)). Perfect moths emerged with 6 and 4 mg./g. of linoleic acid. With 2 and 1·3 mg. the wings became more and more defective. Few moths emerged with 0·33 mg. and none in the diet without linoleic acid. In the same test 4 mg. linoleic acid, without the unsaponifiable fraction, had no effect whatsoever (Table 2 (3)). This test was carried out simultaneously with the corresponding test on *E. kuehniella*, and it is important to note that, unlike *kuehniella*, there was no obvious detrimental effect on growth in the diets with the higher quantities of linoleic acid. Speed of growth was rather variable, but showed no tendency to acceleration or delay according to the quantities of linoleic acid, and it was of the same order on diets without it.

A very similar result was obtained in tests with graded doses of linoleic acid and with vitamin E in place of the unsaponifiable fraction (Table 2 (6)). These tests were conducted at an earlier stage, before our classification of scale deficiences had been worked out, and early stages of deficiency may have been overlooked. It is almost certain that down to 3·3 mg. of linoleic acid the wings were normal. With 1 mg. they showed varying degrees of nakedness and hardly any moths emerged with 0·33 mg. (Table 2 (6*e*)). There was again no indication that linoleic acid had any effect, favourable or unfavourable, on growth. With 4 mg. of linoleic acid, and without vitamin E, none of the moths emerged, but unexpectedly growth was considerably faster than in all the other diets with linoleic and vitamin E. Some tests were also carried out with diets which contained ether extracted yeast. Here again, 5 mg. linoleic acid accelerated growth, and while normal moths appeared in the presence of vitamin E, none emerged in its absence (Fig. 6).

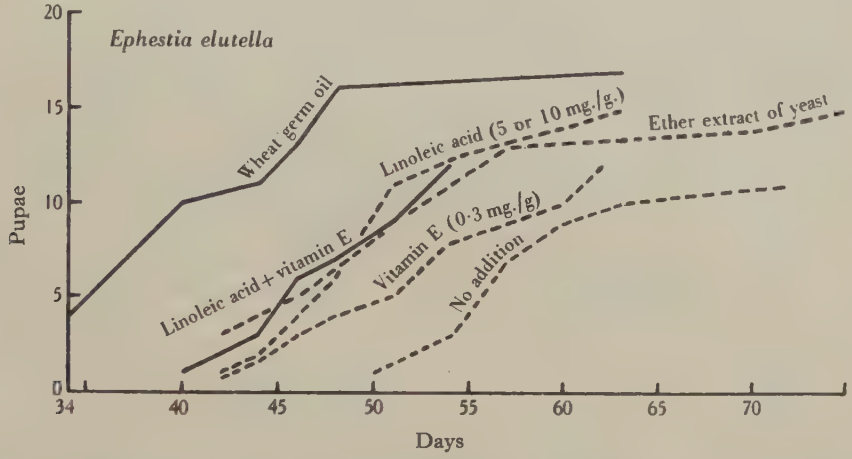

Fig. 6. Growth of *Ephestia elutella* on artificial diets in the presence of linoleic acid and/or vitamin E or the ether extract of yeast. All diets contain yeast which had been thoroughly extracted with ether.

D. *The unsaponifiable factor*

Wheat-germ oil contains relatively large amounts of unsaponifiable matter, most of which is probably sitosterol. The sample which we used in the latter parts of our experiments contained 8% of unsaponifiable matter. It is now known that insects require sterols in the diet for growth (Fraenkel & Blewett, 1943), and to meet these requirements all the diets dealt with in this paper contained 1 % of cholesterol. There still remained a possibility that specific sterols in the unsaponifiable fraction of wheat-germ oil had an effect on the growth of *Ephestia*. Some tests were performed with several preparations of varying purity from wheat-germ oil (prepared by Glaxo Laboratories), and although the results were somewhat ambiguous it is almost certain that sterols play no part in the growth-promoting activity of the unsaponifiable fraction of wheat-germ oil.

From the results given in the foregoing section it is clear that the unsaponifiable fraction stabilizes linoleic acid. The unsaponifiable fraction of wheat-germ oil contains large amounts of vitamin E, and in view of the well-known function of vitamin E as an antioxidant, tests were performed with vitamin E in place of the unsaponifiable fraction. Two samples of tocopherols were used, *dl*-α-tocopherol from Glaxo Laboratories ('synthetic and probably well over 95 % pure') and α-tocopherol acetate from Roche Ltd. These two samples did not always give exactly the same results, but it is not certain whether such differences were significant. When vitamin E was added to the saponifiable fraction of wheat-germ oil, the growth rate of *E. kuehniella* improved, but the extent of this improvement varied on different occasions. This may have been due to difficulties of dosage and to the instability of α-tocopherol. A series of tests with diets containing the saponifiable fraction of wheat-germ oil and graded doses of vitamin E, showed clearly an optimum effect with 0·31 mg. of vitamin E per g. of the diet (Fig. 7). With this concentration growth was greatly speeded up, much more so in this particular test than by the unsaponifiable fraction of wheat-germ oil. Larger doses (1·5 mg.) had a detrimental effect on growth, and with smaller doses (0·06 and 0·01 mg.) growth was also slower (Fig. 7). 0·33 mg./g. were subsequently used in most tests, but it must be remembered that with the narrow range of optimal efficiency and the well-known instability of α-tocopherol, optimal conditions cannot be expected to prevail in every experiment.

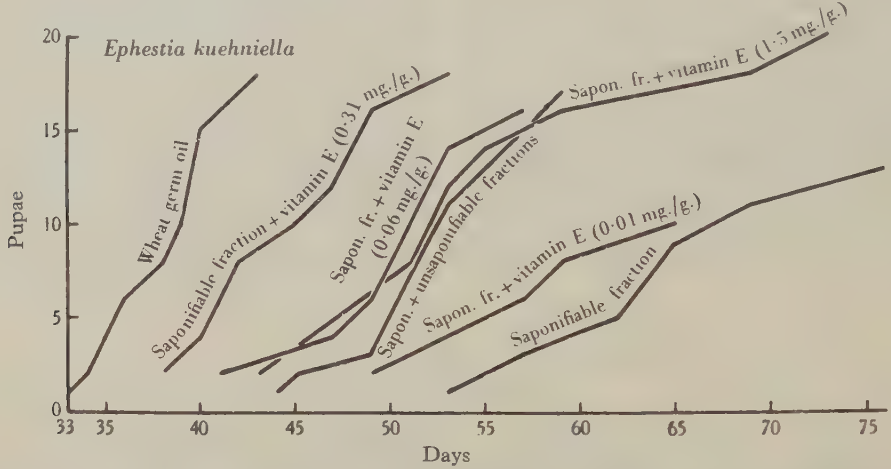

Fig. 7. Growth of *Ephestia kuehniella* on artificial diets in the presence of the saponifiable fraction of wheat-germ oil and graded doses of vitamin E.

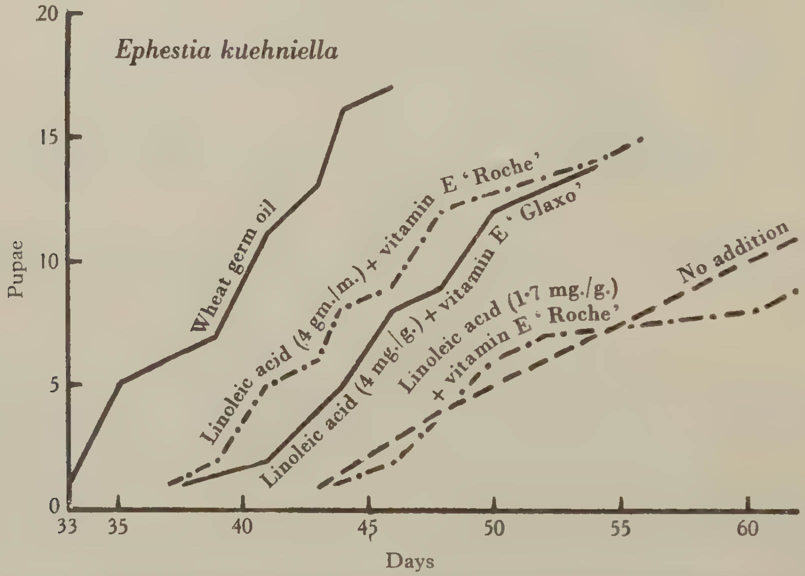

Fig. 8. Growth of *Ephestia kuehniella* on artificial diets in the presence of linoleic acid and vitamin E.

The amount of vitamin E in two samples of wheat-germ oil used by us was kindly estimated by Messrs Vitamins Ltd. and found to be 0·23 and 0·3 %, calculated as α-tocopherol. The amount present in a satisfactory diet, with ½–1 % wheat-germ oil, is therefore very much smaller than the amounts necessary when pure α-tocopherol is used. This is not surprising in view of the labile nature of this compound.

Subsequently, the effect of vitamin E was tested in combination with pure linoleic acid (Fig. 8). It will be remembered that with linoleic acid alone the moths either failed to emerge, or emerged with defective wings (Table 1 (3)). Perfectly formed moths emerged with 0·33 mg. vitamin E (Glaxo) and 4 mg. linoleic acid (Table 1 (5)), and growth was considerably better (Fig. 8) than in the corresponding test with the same quantity of linoleic acid and the unsaponifiable fraction of wheat-germ oil (Fig. 4). With vitamin E (Roche) the moths emerged with defective wings (Table 1 (4)), but growth was again better than with the unsaponifiable fraction of wheat-germ oil, where the moths had been perfect. Vitamin E, in this experiment, therefore, had as good or almost as good an effect on the scale condition as the unsaponifiable fraction of wheat-germ oil, and a better effect on growth. This latter effect may perhaps be attributed to the larger amounts of vitamin E in the diets containing pure α-tocopherol. There can be therefore little doubt that most, if not all, of the favourable effect of the unsaponifiable fraction in the presence of the saponifiable fraction or linoleic acid is due to its content of vitamin E.

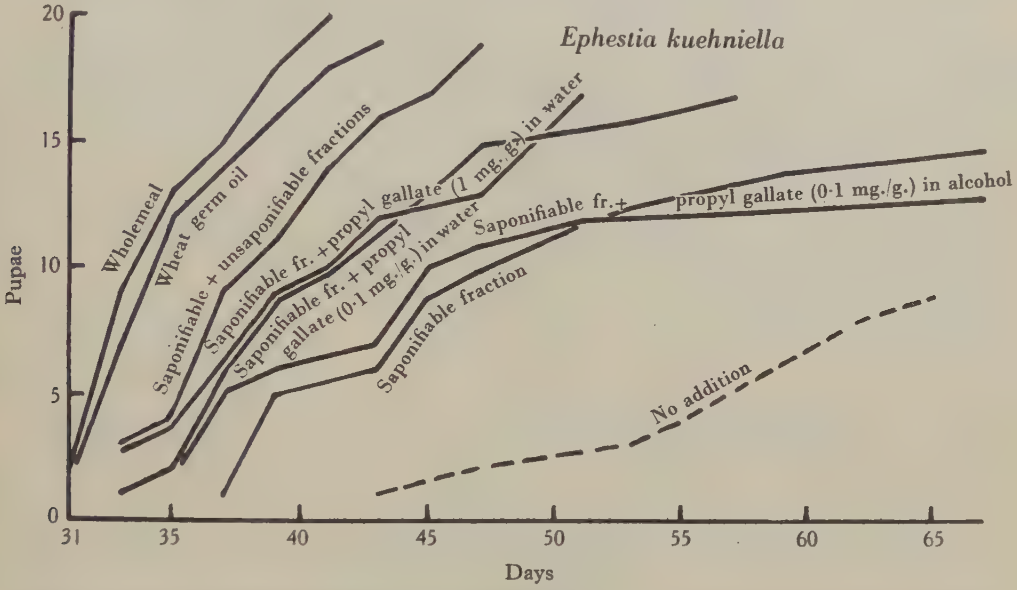

Fig. 9. Growth of *Ephestia kuehniella* on artificial diets in the presence of the saponifiable fraction of wheat-germ oil and propylgallate.

Tests with *E. elutella* on the effect of vitamin E in combination with linoleic acid have already been described. The efficiency of vitamin E in stabilizing linoleic acid seems to be about the same as that of the unsaponifiable fraction of wheat-germ oil (Table 2).

The effect of vitamin E so far described raises the question of its specificity as an antioxidant. It was of interest to see whether other antioxidants had a similar effect in the nutrition of *Ephestia*. Tests with hydroquinone were unsuccessful, owing, possibly, to a toxic action. There was, however, a pronounced positive effect with gallates. Two preparations were used, ethylgallate (B.D.H.) and propylgallate (Nipa Laboratories), and the results obtained with both were similar. Whenever gallates were added to a diet which contained the saponifiable fraction of wheat-germ oil, there was a conspicuous improvement in the growth rate, but the acceleration was smaller than with the unsaponifiable fraction (Fig. 9). This improvement was most striking with large quantities of gallates (*c.* 0·1 % of the diet, and 10 % of the fat) which were incorporated in the diet in aqueous solution. With smaller quantities (0·01 % of the diet) the effect was better when the gallates were added in aqueous solution than when incorporated in the saponifiable fraction in alcoholic solution.

As a third type of antioxidant, ascorbic acid was added to a diet which contained the saponifiable fraction of wheat-germ oil. It was mixed into the diets in aqueous solution. With 0·33 mg. of vitamin C growth of *E. kuehniella* was slightly improved, while a ten times larger quantity seemed to have a detrimental effect (Fig. 10).

The question which remains to be answered is whether the antioxidant action of the unsaponifiable fraction, or vitamin E, on linoleic acid is the sole function of these substances in the nutrition of *Ephestia*. This does not seem very probable in view of the fact that in the absence of the saponifiable fraction the unsaponifiable fraction in most cases pronouncedly speeds up growth. Therefore the effect of adding vitamin E alone was investigated. Vitamin E on each of three occasions markedly improved growth of *E. kuehniella* to a very similar extent as the unsaponifiable fraction, and the moths, as one would have expected, failed to emerge from the pupae (Fig. 10). *E. elutella*, as stated above, sometimes grows relatively fast even in the absence of wheat-germ oil, and very much slower if the yeast in the diet has been extracted with ether. Vitamin E, added to a diet with extracted yeast, accelerated growth though not as much as did the ether extract of yeast (Fig. 6).

The effect of ethylgallate in the absence of the fat fraction of wheat-germ oil has so far been tested only once (1 mg./g.) and there was a slight improvement in the growth rate. The suggestions had been made above, in connexion with tests with graded doses of wheat-germ oil, that a technical sample of petrol ether, which had been used as a diluent, may have had an effect of its own on growth. Subsequently it was added to diets in similar amounts (1·3–2 mg./g.), but in the absence of any oil or oil fraction, and on each of two occasions had a slightly favourable effect on growth (Fig. 10).

All the evidence so far available therefore points to the effect that certain antioxidants have a favourable influence on the growth rate of *Ephestia*, quite independent of the stabilizing effect which they have on dietary fats.

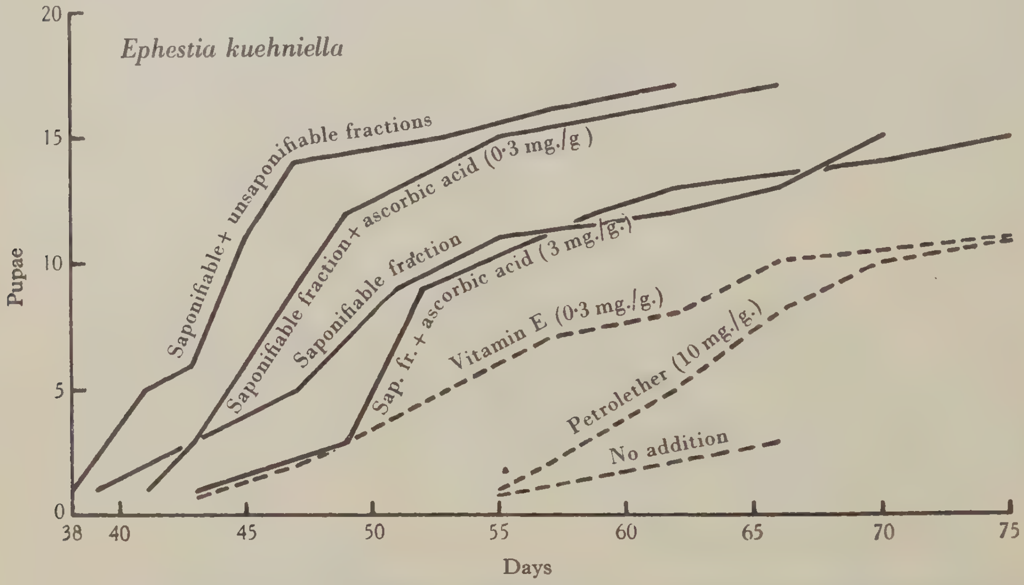

Fig. 10. Growth of *Ephestia kuehniella* on artificial diets in the presence of the saponifiable fraction of wheat-germ oil and/or ascorbic acid, vitamin E or petroleum ether.

Table 3. *The effects of various natural fats on growth, and the state of scales in the wings of* Ephestia kuehniella

	Effect on		Linoleic acid content
	Growth	State of scales	
Wheat-germ oil	Good	Good	50–60 % (Hilditch, 1940)
Coconut oil	None	None	1–2 % (Hilditch, 1940)
Allenblackia oil	None	None	0·2 % (Hilditch, 1940)
Yeast fat	Doubtful	None	4 % (Hilditch, 1940)
Cod-liver oil	Good	None	Absent (Farmer *et al.* 1938*a, b*)
Halibut-liver oil	Good	None	Absent (Lovern, 1932)
Lard	Good	Slight	5–10 % (Hilditch, 1940)
Butter fat	Good	Slight	3–5 %* (Hilditch, 1940)

* Geometrical isomerides.

E. *The effect of adding oils from different sources*

So far, we have dealt mainly with the effect of wheat-germ oil, or of fractions or substances derived from or contained in it. In the following section we shall describe the effects of fats from other sources.

In the summary of these tests, given in Table 3, it can be seen at a glance that the effect on the scale condition is very much dependent on the amount of linoleic acid which these fats contain. The effect on growth is very different with different fats as one would have expected, since the fats used probably differ very much in the degree of refinement, freshness and stability. Table 3 requires some explanations.

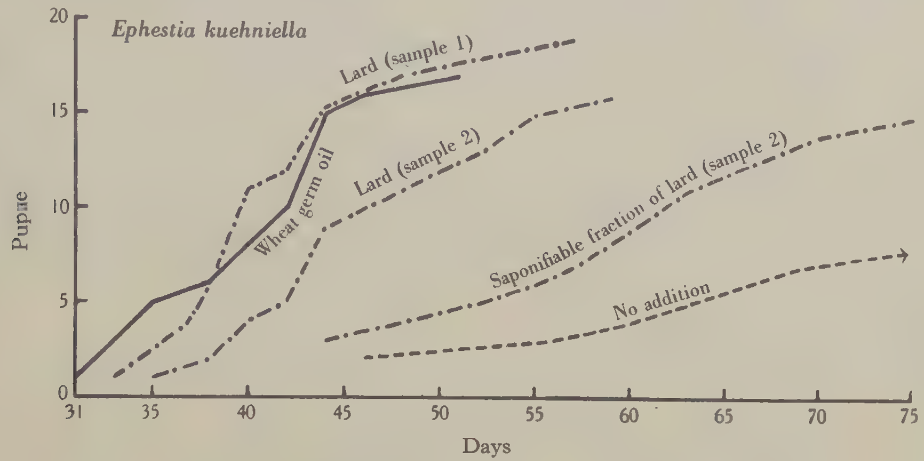

Fig. 11. Growth of *Ephestia kuehniella* on artificial diets with the addition of lard or the saponifiable fraction of lard.

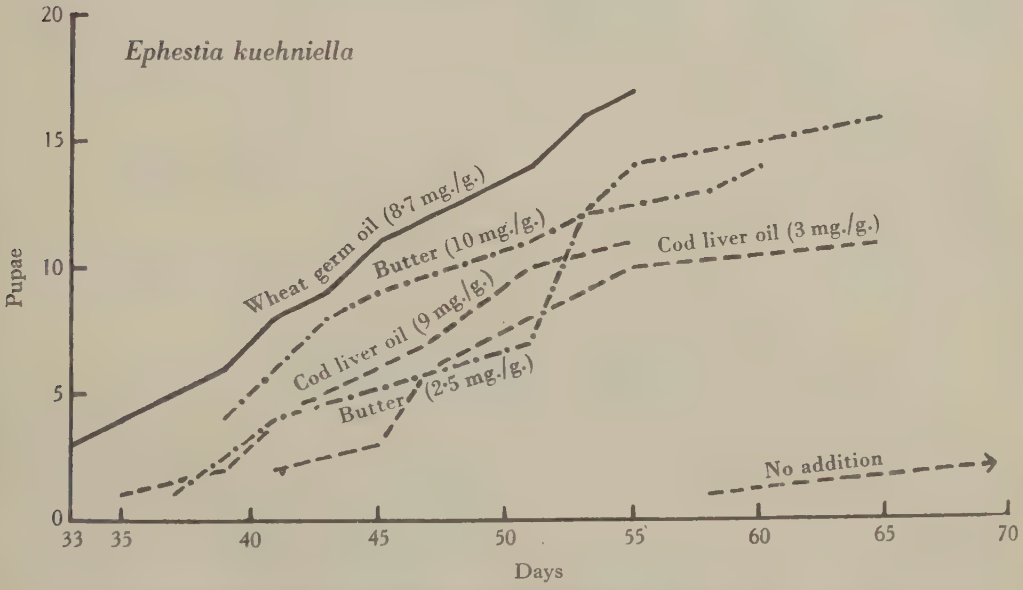

Fig. 12. Growth of *Ephestia kuehniella* on artificial diets in the presence of butter or cod-liver oil.

(1) *Lard.* The sample used was prepared from fresh pig fat by mincing, boiling in water and straining through cloth. The linoleic acid content of our samples are not known. According to Hilditch, Lea & Pedelty (1939) linoleic acid in lard is derived entirely from the food, and typical examples contain 3–8 %. The moths which emerged from diets containing lard invariably had partly naked wings, but the exact state was not investigated at the time (Table 1 (13)). The results obtained would be consistent with a linoleic acid content of the lard of *c.* 7 % (the effect of 10 mg. lard being approximately the same as that of 1·2 mg. of wheat-germ oil). While lard, therefore, proved a very moderate source for linoleic acid, it had a very pronounced effect on growth which in the best cases was as fast as on wheat-germ oil (Fig. 11). Lard contains *c.* 2 % of highly unsaturated C_{20-22} acids which include arachidonic acid (Hilditch *et al.* 1939). From our results with cod-liver oil (see below) it is not impossible that these acids may improve growth. The saponifiable fraction

of lard also improved growth, but to a lesser degree than whole lard. Lard is usually considered to be poor in natural antioxidants. It contains relatively little unsaponifiable matter, and we have not succeeded in obtaining sufficient for a test.

The effect of lard on *Ephestia* closely resembles that on rats, where the skin condition is affected according to its linoleic acid content, while the effect on growth is also disproportionally superior (Hume, Nunn, Smedley-Maclean & Smith, 1938).

(2) *Cod-liver oil and halibut-liver oil* had a very pronounced growth effect (Figs. 12, 13) similar to lard, but the effect on the scales was entirely negative (Table 1 (10, 11)). Most moths failed to emerge, and those which did emerge were very badly formed. This fully agrees with what is known about the composition of these oils. According to Farmer & Van den Heuvel (1938*a*, *b*) cod-liver oil contains small amounts of polyethenoid acids with the following numbers of ethenoid linkings: C_{16}, 3; C_{18}, 4; C_{20}, 5; C_{22}, 6. Ordinary linoleic acid has not been detected. Halibut-liver oil contains no linoleic acid (Lovern, 1932). The fact that these two oils had no effect on the scale conditions made it the more surprising that there should have been such a good effect on growth. As this result very closely resembled that of similar work with rats (Burr, Burr & Brown, 1931; Burr, Brown, Kass & Lundberg, 1940; Hume *et al.* 1938) tests were carried out with different fractions from cod-liver oil, prepared by the method of fractional distilling and kindly put at our disposal by Dr E. H. Farmer. Of the various samples tested, a striking effect on growth was obtained with the pure methyl ester of docosahexenoic acid (Fig. 14). With 2·5 mg. of this acid, and in the presence of the unsaponifiable fraction of wheat-germ oil, there was a very considerable improvement in the growth rate if compared with the effect of the unsaponifiable fraction alone (which on this particular occasion had relatively little effect on growth). Four times this quantity of the C_{22} acid had an inferior effect. Growth was not improved when this acid was supplied alone or together with the saponifiable fraction of wheat-germ oil, which clearly indicates that the C_{22} acid is liable to deteriorate unless stabilized by the unsaponifiable fraction. At the same time the C_{22} acid had no effect whatsoever on scales or emergence. With the methyl ester of the unsaturated C_{20} acid from cod-liver oil there was no similar effect on growth when applied in combination with the saponifiable or unsaponifiable fraction of wheat-germ oil, but there was a conspicuous growth effect when C_{20} acids were added alone (Fig. 15). With mixtures of the saturated and unsaturated C_{20} acids and mixtures of the saturated and unsaturated C_{22} acids growth was much improved, and with a mixture of the C_{22} plus higher methyl esters plus some unsaponifiable material slightly improved. These mixtures were tested in the absence of the saponifiable or unsaponifiable fraction of wheat-germ oil, and none of these mixtures had any effect on scales.

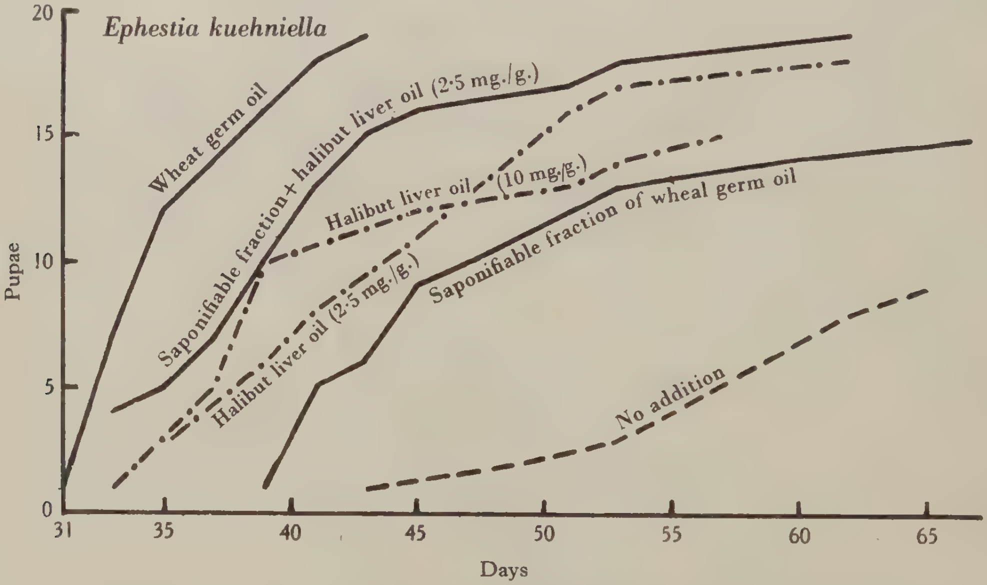

Fig. 13. Growth of *Ephestia kuehniella* on artificial diets in the presence of halibut-liver oil and/or the saponifiable fraction of wheat-germ oil.

It seems therefore fair to conclude that the growth effect of cod-liver and possibly also halibut-liver oil is

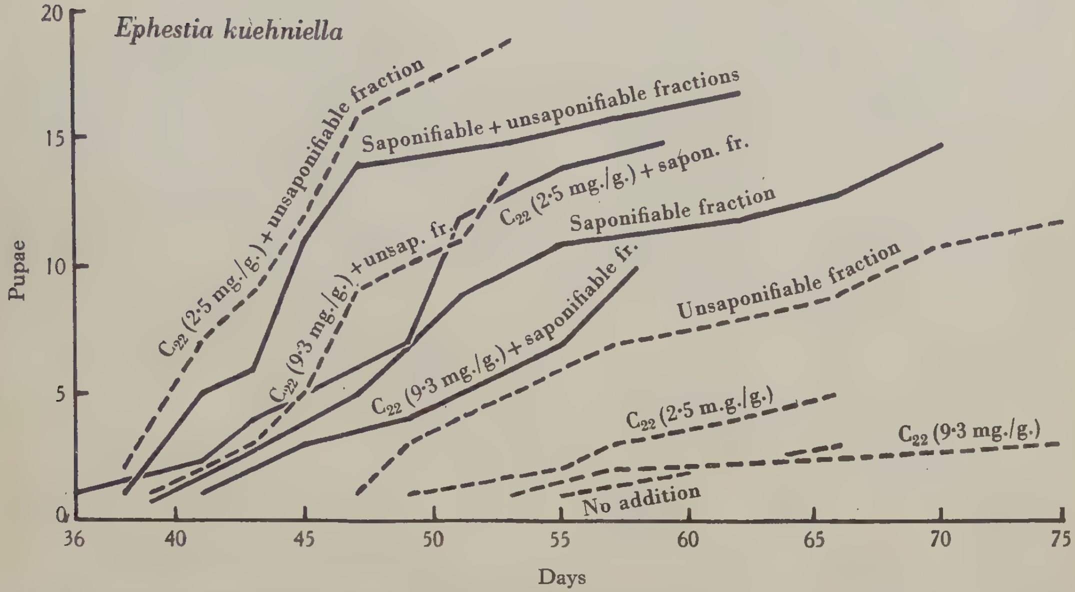

Fig. 14. Growth of *Ephestia kuehniella* on artificial diets in the presence of the C_{22} polyethenoid acid (docosahexenoic acid) from cod-liver oil and/or the saponifiable or unsaponifiable fraction of wheat-germ oil.

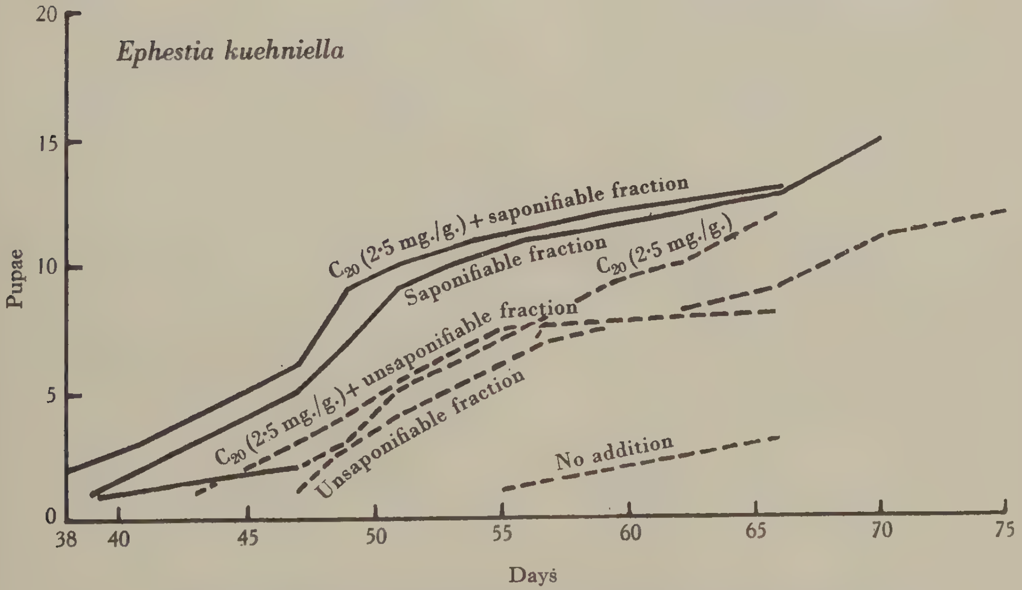

Fig. 15. Growth of *Ephestia kuehniella* on artificial diets in the presence of the C_{20} polyethenoid acid from cod-liver oil and/or the saponifiable or unsaponifiable fraction of wheat-germ oil.

partly, if not entirely, due to its content of docosahexenoic and other unsaturated acids.

(3) *Butter fat* (shop-bought 'National' butter). With 10 mg./g. of butter in the diet all the moths emerged from the pupae, but either badly formed, or with most of the scales missing on the wings (Table 1 (12 *a*)). With 2·5 mg. about half the moths failed to emerge (Table 1 (12 *b*)). The effect of butter therefore seems to be somewhat inferior to that of lard, and consistent with a linoleic acid content of the order of 3 %. According to Hilditch (1940) milk fat contains only traces of ordinary linoleic acid, but 'there is a certain proportion (3–5 % of the total fatty acids) of octadecadienoic acids which appear to be different geometrical (*cis-trans*) isomerides of the $\Delta^{9:10,\,12:13}$-acid'. From the results obtained by us it therefore appears that this isomer is utilized by *Ephestia* to the same degree as linoleic acid from vegetable sources. Rats do not appear to utilize this acid (Burr, 1942).

Butter had a very marked positive growth effect, similar to that of cod-liver oil (Fig. 12). We do not know whether this is caused by this octodecadienoic acid, or antioxidants or something else. A superior growth of rats on diets with butter fat has been attributed to the presence, in butter, of small amounts of long-chain saturated fatty acids (Boutwell, Geyer, Elvehjem & Hart, 1941).

(4) *Yeast oil*. The sample used was obtained from dried brewers' yeast by extraction with ether. There was no effect on the condition of the scales and emergence, neither with *kuehniella*, *elutella* or *cautella*, but there was a conspicuous improvement in growth in certain tests with *elutella* and *cautella* (but never with *kuehniella*) especially where ether extracted yeast was used in the diet (Fig. 6). Yeast oil contains a little linoleic acid (4·1 % according to Teufel, Thaler & Schreyegg, 1936), but it seems feasible that this might have been destroyed during the drying of the extracted oil at about 100° C. This treatment might also have affected unfavourably the antioxidant constituents and this is possibly the cause of the different effects on growth of different samples. Yeast oil is also inefficient in alleviating symptoms of a linoleic acid deficiency in the rat (Burr, 1942).

(5) *Coconut oil* (from Prof. T. P. Hilditch). There was no effect on scale condition with the addition of this oil alone, and a small effect on growth. With coconut oil, and in the presence of the unsaponifiable fraction of wheat-germ oil, most moths of *E. kuehniella* and *elutella* emerged, but either incompletely formed or with the wings naked. The linoleic acid content of coconut oil is given as 1–2 % (Hilditch, 1940), which is consistent with the results obtained on the diets with the unsaponifiable fraction of wheat-germ oil.

(6) *Allenblackia oil* (from Prof. T. P. Hilditch) had an even smaller effect than coconut oil. In the presence of the unsaponifiable fraction of wheat-germ oil some very badly formed moths of *E. kuehniella*, but none of *E. elutella*, emerged, and growth in both species was slightly improved. Without the unsaponifiable fraction no moths emerged. Allenblackia oil is reported to contain only about 0·2 % of linoleic acid (Hilditch, 1940).

F. *Diets which contain starch*

In our early experiments with *E. kuehniella*, long before the effects of wheat-germ oil were recognized, we had noticed that moths invariably emerged from the pupal case in the absence of wheat-germ oil if the diets contained starch in place of glucose. No close examination of the state of scales on the wings was made at the time, but it appears that with 50 % rice starch at least two out of nineteen emerged with bare wings, while the remaining seventeen, and also all the moths from a diet with 80 % starch, seem to have emerged with the wings either intact or not greatly deficient. A subsequent test (Fig. 16) with maize starch (80 %) yielded nine moths, five of which had defective wings. With wheat-germ oil added to this diet growth was considerably speeded up and all moths were normal. A simultaneous test with a sample of potato starch showed very bad growth. Only two pupae were formed and none emerged. With wheat-germ oil growth was still very slow but six perfect moths emerged. On a sample of potato starch which we prepared ourselves from fresh potatoes growth was relatively better, but again the moths only emerged in the presence of wheat-germ oil. In a third series of tests another sample of rice starch was used which had a peculiar flavour and may not have been in perfect condition. Here growth was very slow, both with and without wheat-germ oil, and without the oil altogether five moths emerged, all with the scales missing.

It has been stated before that severe linoleic acid deficiency symptoms in rats do not develop on diets which contain starch (Burr, 1929; Evans, 1932), while deficiency symptoms readily appear if potato starch is used (Burr, 1942). According to Taylor & Lehrman (1926) cereal starches always contain some fat which is difficult to remove; the combined fatty matter in maize starch is about 0·5 %, and in rice starch 0·83 %, with approximately 24 % palmitic, 40 % oleic and 36 % linoleic acid. Lehrman & Kabat (1933) state that potato starch contains no combined fatty matter. The linoleic acid contents of the starches used by us are not known, but assuming them to be of the order of those investigated by Taylor & Lehrman, it would appear that our starch diets contained *c.* 0·2 % of linoleic acid, presumably in a relatively stable form. From what we know about requirements of linoleic acid by *Ephestia*, it seems feasible that these amounts may be sufficient to ensure the formation of some perfectly developed moths, and it does not seem necessary to postulate any special specific effect of starch, such as 'refection'.

DISCUSSION

The investigation began with the discovery that wheat-germ oil was essential for the development of

Ephestia in two ways. The caterpillars failed to grow or grew very slowly on artificial diets in the absence of wheat-germ oil, and those which ultimately pupated failed to emerge as adult moths. This twofold effect of wheat-germ oil on emergence and on growth could be localized in two different fractions: the saponifiable fraction was primarily necessary for emergence and good scales and the unsaponifiable fraction for growth. Further analysis led ultimately to the recognition of linoleic acid as the saponifiable and vitamin E as the unsaponifiable factor. The main complication in the interpretation of the results rests in the fact that these two factors not only show an independent action of their own on growth and scales, but also interactions indispensable for the complete functioning of the mechanism. On the one hand, linoleic acid, to have an effect at all, must be stabilized by vitamin E or other antioxidants and, on the other hand, the growth-promoting effect of vitamin E may be antagonized by the formation of toxic substances in the deterioration of linoleic acid, probably peroxides. These interactions made it difficult at first to recognize that linoleic acid has also a growth-promoting effect of its own.

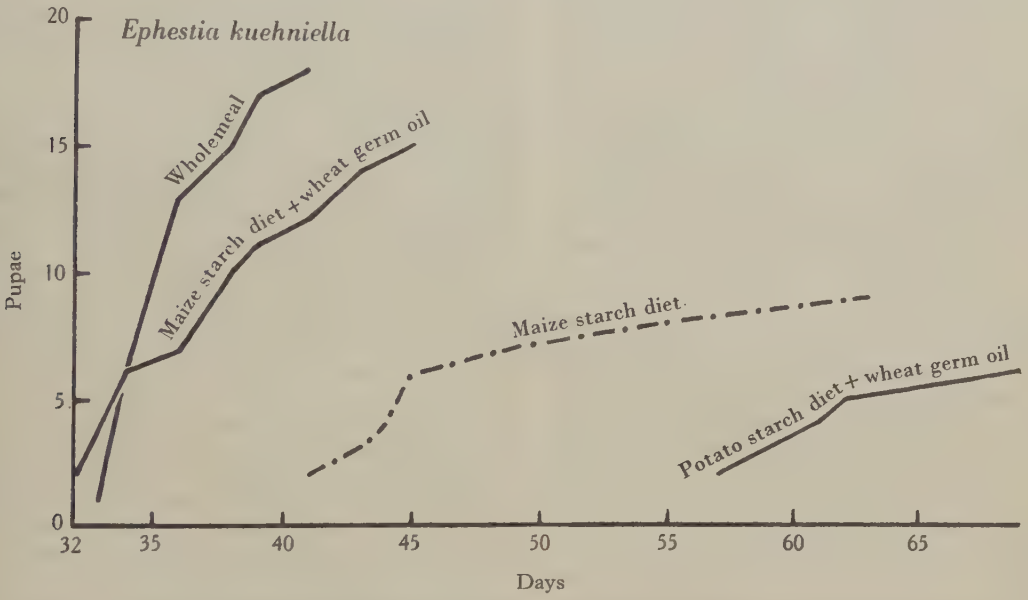

Fig. 16. Growth of *Ephestia kuehniella* on artificial diets which contain maize starch or potato starch.

A. *The effect of linoleic acid on scales and emergence*

There is now overwhelming evidence for the fact that linoleic acid constitutes the saponifiable factor necessary for good formation of scales and emergence. Graded doses of linoleic acid were shown to have an effect on scales exactly similar to that with graded doses of wheat-germ oil. With different oils the effect is closely parallel to their linoleic acid content. Further evidence is supplied by the positive effect of cereal starch, which, as is already known, usually contains small quantities of linoleic acid. Furthermore, linolenic acid has an identical or similar effect.

The exact physiological mechanism by which linoleic acid works is not yet known. All the known facts indicate that it is connected with the production or action of the moulting fluid. An insect during moulting or emergence from the pupal case excretes a fluid into the space between the old and new cuticle which facilitates the easy separation of the two. It is easy to see that the amount or the constitution of this fluid might influence emergence as shown by *Ephestia*. Without linoleic acid there is no emergence, with very little linoleic acid the cuticles separate, but the scales remain stuck to the wrong cuticle, and with more and more linoleic acid more and more scales remain in the right position. All we know about the development of the Lepidoptera wing in general, and of the wing of *Ephestia* especially, is compatible with this explanation (Köhler, 1932).

B. *Stabilizing effect of vitamin E on linoleic acid*

It is well known that linoleic acid easily deteriorates by rancid oxidation, with the formation of toxic peroxides (Burr & Barnes, 1943 *a*). This fully explains why linoleic acid has little or no effect, except in combination with the unsaponifiable fraction, vitamin E or other antioxidants. Perhaps the clearest example of this relationship is provided by the case where large quantities of linoleic acid produced perfect scales but bad growth, while smaller quantities produced bad scales and better growth. Obviously there had been some deterioration in the linoleic acid; with larger quantities more toxic substances were formed, while sufficient effective linoleic acid was retained. The antioxidant effect of vitamin E on linoleic acid has been mainly studied in connexion with oxidation of vitamin A by unsaturated fatty acids and its protection by vitamin E (Thompson & Steenbock, 1944; Dam, 1943, 1944; György & Tomarelli, 1944).

C. *Effect of linoleic acid on growth*

Considering that linoleic acid, in the absence of suitable antioxidants, easily deteriorates with the production of deleterious products, it is not surprising that growth in such cases is slow. This effect could easily be taken to indicate that the effect of linoleic acid was primarily concerned with scales, and not with growth and, moreover, would mask any active growth-promoting effect which linoleic acid might possess. There have been, in fact, several indications which make the assumption of such a growth-promoting effect inevitable. On certain rare occasions linoleic acid, when added alone, or in combination with the unsaponifiable fraction or vitamin E, had a marked positive effect on growth, and such positive effects must have some significance. Again, linolenic acid, which obviously acts similarly to linoleic acid, had a pronounced growth effect, both when given alone and in combination with the unsaponifiable fraction. There have been many occasions when growth was faster in the presence of the saponifiable and unsaponifiable fractions than with unsaponifiable alone. Finally, it is conceivable that the growth-promoting effect of the highly unsaturated C_{20} and C_{22} acids from cod-liver oil and possibly also from lard is essentially of the same nature, while it is impossible to attribute it to any antioxidant action. In fact, the pro-oxidant effect of these acids has been frequently noted in connexion with a destructive effect on vitamins A and E (Mattill & Golumbic, 1942; Dam, 1944).

D. *Effect of vitamin E and other antioxidants on growth*

Apart from the protective action on linoleic acid, vitamin E and probably other antioxidants also have a positive effect on growth in the absence of linoleic acid. This is most clearly shown by the effect of the unsaponifiable fraction alone on growth. Positive effects of a smaller order were also produced with pure α-tocopherols or gallates. In view of the labile nature of these substances and the toxic nature if given in excess, it is not surprising that the pure substances should have had a smaller effect than when given in the unsaponifiable fraction. Furthermore, the fact that in the presence of ample linoleic acid growth is sometimes very bad may be taken to indicate that vitamin E is being destroyed while exerting its antioxidant effect, an explanation given for the phenomenon that cod-liver oil may be the cause of a vitamin E deficiency (Mattill & Golumbic, 1942; Dam, 1944; Clausen, Barnes & Burr, 1943). The idea that an antioxidant should have an effect of its own, independent of the presence of dietary fat, is not entirely new. The stability of adipose fat of rats, for instance, is dependent on the presence of antioxidants, such as vitamin E, which are entirely of dietary origin (Barnes, Lundberg, Hanson & Burr, 1943). Insects contain relatively large quantities of fat in the fat body, and it is not difficult to imagine that the presence of antioxidants, originally derived from the food, may be of advantage expressed by a better growth rate. The effects of vitamin E deficiency in vertebrates are manifold, and it has been suggested that they may all be connected with the antioxidant function of vitamin E (*Nutrition Reviews*, 1945).

All our results can therefore be explained by assuming that linoleic acid is necessary for good scales and good growth, while vitamin E is necessary for protecting linoleic acid, and, in addition, for good growth. In view of the highly labile nature of these substances it would have been surprising if the results obtained on different occasions had been exactly identical. Moreover, the conditions under which the tests were performed were highly unfavourable for work with such labile compounds. Very small amounts of these substances were evenly distributed in large amounts of casein and glucose. The diets were finely ground, air dried, and allowed a free circulation of air. They were kept unchanged for at least 4–6 weeks in the case of favourable diets, and much longer with unfavourable ones. In these circumstances the conditions for oxidation of oxidizable substances are obviously very favourable. It would, of course, be preferable to change diets at frequent intervals. Unfortunately, such a course is impracticable, if not impossible, with *Ephestia*, as the larvae are hidden in a tangle of webbing and food and cannot easily be extracted without being damaged.

The effect of linoleic acid on rats, as shown by the work of Burr and Smedley-Maclean, is strikingly similar in many respects to the effect on our moths. The effect is mainly concerned with the skin; it is probably a coincidence that rats develop 'scaly' skins, while it is 'scales' on the wings which are affected in moths. Linoleic acid also shows a growth effect. Lard improves the skin condition according to the quantity of linoleic acid it contains, while there is a disproportionately superior effect on growth. The most remarkable agreement concerns the positive effect of cod-liver oil on growth, but not on scales, which appears to be to some extent due to the presence of docosahexenoic and other highly unsaturated acids. Linoleic acid deficiency does not develop fully in the presence of cereal starches, but it does with potato starch. The magnitude of requirements was difficult to determine in both cases. For rats it amounts to about ½–1 % of the diet (Burr & Barnes, 1943 *b*), while for *Ephestia* it seems to be ¼–½ %. There are also certain discrepancies. Linolenic acid, according to most observers (Hume *et al.* 1938; Quackenbush, Kummerow & Steenbock, 1942), has a very inferior effect on growth and skin of rats, while for *Ephestia* it is at least equal to linoleic acid. Isolinoleic acid from butter has no effect on rats (Burr, 1942), while for *Ephestia* it seems to be as good as ordinary linoleic acid.

From the little known about the composition of insect fats (Hilditch, 1940) it is not possible to decide whether linoleic acid is a constant constituent. Very recently it was shown by Pepper & Hastings (1943) that the linoleic acid content of the fat of the sugar beet webworm, *Loxostege sticticalis* L., decreases during metamorphosis from 26 % to less than 1 %, while oleic acid increases from 31·5 to 54·5 %. This clearly indicates that linoleic acid may be derived from the food and is not synthesized. We have so far failed to obtain any indication that linoleic acid is required by any insect other than the three *Ephestia* species. Even the closely allied species, *Plodia interpunctella*, which occurs together with *Ephestia* species on the same food does not require it. In the clothes moth, *Tineola bisselliella*, wheat-germ oil has a minute positive effect on growth (Fraenkel & Blewett, 1946 *a*). We have grown a number of beetle larvae belonging to the genera *Tribolium*, *Tenebrio*, *Dermestes*, *Lasioderma*, *Sitodrepa* and *Ptinus* in the complete absence of even traces of fat without any indication that fat would have improved growth.

Finally, mention must be made of a genetical aberration in *Ephestia kuehniella*, described by Kühn & Henke (1929) under the term *Glasflügeligkeit*, which appears to be morphologically indistinguishable from our nutritional *Glasflügeligkeit*. The variation series showing the degree of *Glasflügeligkeit* (Kühn & Henke, 1929, fig. 30, p. 76) is indistinguishable from our series obtained with graded doses of wheat-germ oil or linoleic acid, and in both cases the missing scales remain behind on the inside of the pupal case. This makes it probable that the physiological action of this particular gene is concerned with linoleic acid metabolism.

SUMMARY

1. The larvae of the moths belonging to the genus *Ephestia*, *E. kuehniella*, *E. elutella* and *E. cautella* grow well on artificial diets which also contain wheat-germ oil. In the absence of wheat-germ oil growth is slow, the mortality high and the moths fail to emerge from the pupae.

2. With suboptimal quantities of wheat-germ oil moths emerge with the wings lacking in scales.

3. The saponifiable fraction of wheat-germ oil is necessary for emergence and good scales, and the unsaponifiable fraction for good growth.

4. The active substance in the saponifiable fraction is linoleic acid. Linolenic acid has approximately the same effect as linoleic acid. Oleic acid has no effect.

5. The active substance in the unsaponifiable fraction is vitamin E (α-tocopherol) and possibly other antioxidants. One of the functions of vitamin E is that of an antioxidant which stabilizes linoleic acid. In this respect vitamin E can be replaced by ethyl and propyl gallates, or by ascorbic acid.

6. Apart from this stabilizing effect on linoleic acid, the unsaponifiable fraction of wheat-germ oil, or vitamin E, have also an independent growth effect. There are also indications that linoleic acid has a growth effect.

7. It is shown that the effect of a number of vegetable and animal fats on scales and emergence is strictly parallel to their linoleic acid content.

8. With cod-liver oil, halibut-liver oil, lard and butter growth is fast, while the effect on scales and emergence is small or nil. The growth effect of cod-liver oil is mainly or partly due to its content of docosahexenoic and probably other highly unsaturated fatty acids.

9. Large quantities of cereal starches in the diet have a favourable effect on scales and emergence which is attributed to the presence, in starch, of impurities of linoleic acid. No such effect has been encountered with potato starch which is free of linoleic acid.

10. The similarities and dissimilarities in the linoleic acid deficiency of the rat and *Ephestia* have been discussed.

11. The symptoms of the linoleic acid deficiency of *Ephestia* are similar, if not perhaps identical, with those of a genetical condition known as *Glasflügeligkeit*.

We gratefully acknowledge a grant by the Medical Research Council for the study of insect nutrition, of which this investigation is a part. We are also indebted to Prof. T. P. Hilditch (Liverpool) for analyses of wheat-germ oil and supplies of coconut and Allenblackia oil, the late Mrs I. Smedley-Maclean (Lister Institute) and Prof. G. O. Burr (Minneapolis) for gifts of linoleic acid, Dr E. H. Farmer (British Rubber Research Association) for gifts of linolenic acid and of docosahexenoic acid and other fatty acids isolated from cod-liver oil, Dr G. King (St Mary's Hospital) for gifts of oleic and elaidic acid, Vitamins Ltd. for a gift of wheat-germ oil and the analysis of its vitamin E content, and Glaxo Laboratories Ltd. for a gift of α-tocopherol.

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VOLATILE ACID IN THE DIGESTA OF RUMINANTS AND OTHER ANIMALS

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(With One Text-figure)

A mixture of volatile organic acids, consisting for the most part of the fatty acids, acetic, propionic and butyric, is a characteristic product of fermentation within the alimentary tract. It was shown previously that volatile acids are absorbed directly from the organ in which they are produced (Barcroft, McAnally & Phillipson, 1944), and this fact, together with a consideration of the quantities present in the digesta—a subject with which the present paper is concerned—suggest that they form a valuable part of the calorific material utilized by the animal.

The literature provides little information concerning the quantity of organic acids present in the rumen, and this work was undertaken primarily to fill this gap. It was subsequently extended to include the whole digesta, not only of the ruminant but also of the horse, pig, rabbit and rat, in order to find out whether fermentation, as far as can be judged by the quantity of volatile acid present in the digesta, takes place to the same extent in these animals as it does in the ruminant. The results show that the quantity of volatile acid in proportion to the body weight is greatest in the ox and least in the rat. The quantity present in each animal, however, is to a large extent dependent upon the quantity of digesta present in the alimentary tract, for the difference in concentration of volatile acid in proportion to the digesta shows less variation.

METHOD

General procedure

The alimentary tract was divided by ligatures into stomach, small intestine, caecum, colon and rectum, as soon as possible after death. Each portion was weighed before and after removing the digesta. Volatile acid was estimated by alkali titration of the steam distillate, and the results were expressed as grams of acetic acid either per 100 g. dry matter or as the total quantity present in the digesta.

Four sheep, two oxen, one red deer, three horses, four pigs (8–10 weeks old), five rabbits and seventeen rats divided into three batches were used. The animals received the rations specified below and food was not withheld prior to slaughter:

Sheep.	Pasture grass.
Oxen.	Bran and oats, cake, mangolds and hay.
Red deer.	Highland grazing, largely heather.
Horses.	Pasture grass in two cases; straw and mangolds in the third.
Pigs.	Bran.
Rabbits.	Bran and oats and mangolds.
Rats.	Bran and oats and hay.

In experiments, in which material was obtained from the slaughterhouse, large samples were taken from each portion of the digesta and placed in screw-top bottles. These were brought to the laboratory for final sampling. When it was not possible to examine material within a few hours, samples were placed in previously weighed vessels containing normal sulphuric acid in order to prevent further fermentation. This precaution was found to be unnecessary with material that stood for only a few hours before further treatment.

Division of the alimentary tract

Some modifications of the divisions of the alimentary tract stated above were necessary. In ruminants the stomach was divided into three portions: (1) rumen and reticulum, (2) omasum, (3) abomasum. In all the larger animals the small intestine was divided into two parts, the oral two-thirds, corresponding roughly to the duodenum and jejunum, being the first, and the caudal third, approximating to the ileum, being the second.

In all animals the caecum was taken as a separate division of the large intestine. In the ruminants and in the pig, where there is no distinct anatomical division, other than the entrance of the ileum, between the caecum and the colon, an arbitrary division was made by placing a ligature at the spot where the oral portion of the colon comes into close proximity with the caudal portion to form an elliptical coil (Ansa spiralis). This division, instead of the usual one in which the caecum and colon are divided at the ileo-caecal orifice, appeared to be justified on account of the consistency of the digesta, for little difference can be found until the material reaches the spiral part of the colon.

The colon in the ruminant, horse and pig was divided into two parts. The distinction between these two parts is obvious, for in these animals the colon is in the form of a loop, the two sides of which are in close apposition. In the ruminant and pig this loop is in the form of a spiral, while in the horse it is reflexed back at the diaphragm, so that it is divided into four parts. The apex of the colonic loop was taken as the boundary between the first and second parts of the colon.

The rectum included all the large intestine caudal to the colonic loop in the ruminant, pig and horse and so incorporated what is usually called the distal or floating colon. In the rabbit and rat the rectum was taken as commencing where the sacculation of the intestinal wall ceased.

These divisions of the large intestine, although not based on anatomical or zoological criteria, are justified when the consistency of the digesta is considered and may be thought of as physiological.

Live weights of animals

In the cases of the oxen and the horses, it was impossible to weigh the animals either before or after slaughter. With the oxen, the carcass weight was obtained and the live weight estimated on the assumption that the carcass weight represents 54 % of the live weight. The live weight of the horses was estimated by the slaughterman, but, to obtain a better basis for calculating the live weights of the horses, the total weight of the carcass, hide, feet, blood, and the viscera of six horses was obtained after slaughter. The proportion of the weight due to the stomach and intestines filled with digesta was then calculated.* The average figure for the six horses was 18·6 %, the range being from 17·2 to 20·4 %. Using this average figure to calculate the live weights of the horses actually used, figures are obtained which are in all cases higher than those estimated by the slaughterman. Horse 1 has a calculated live weight of 1746 lb. as compared to an estimated one of 1456 lb. The corresponding figures for horse 2 are 1504 and 1008 lb., and for horse 3, 500 and 350 lb. In the case of horse 3, only the weight of the digesta was available to form a basis of calculation, but, taking an average figure from horses 1 and 2, for the proportion of the full stomach and intestines that was due to digesta, namely, 66 %, a figure which deviates from the two directly measured by ± 10 %, the live weight has been calculated at 500 lb. The live weights given in this paper for oxen and horses, therefore, at the best are intelligent guesses.

CHEMICAL METHODS

(*a*) *Dry matter*

Weighed samples were placed in porcelain dishes and dried in a steam oven to constant weight.

(*b*) *Volatile acid*

Weighed samples were transferred to a measuring cylinder and diluted with from five to ten times their weight of tap water. The material was shaken until the digesta were evenly distributed throughout the water, and the solid matter was then strained off through muslin. 50 ml. of the liquid obtained (that is, one-tenth of the original sample in the cases where the dilution was to 500 ml.) were added to 50 ml. of saturated and acidified magnesium sulphate solution, as described by McAnally (1944), and allowed to stand overnight. The precipitate was filtered off and 10 ml. of the filtrate were distilled in the Markham apparatus. Distillates were titrated with 0·01 or 0·02 *N* potassium hydroxide under carbon dioxide free conditions using phenol red as the indicator.

(*c*) *Sources of error*

No account was taken in the calculations of the volume occupied by the dry matter when making the sample up to a definite volume with water. By determining the volume of dry matter, it was found that on an average 1 g. of dry matter occupied 1 ml. If this factor is not allowed for, then the error in the calculations is 2 % for every 10 % dry matter. Thus in material that contains 10 % dry matter the figures given are too high by 2 %; in material containing 20 % dry matter they are too high by 4 %, and so on whenever the dilution is five times. As it is seldom the case, apart from digesta in the rectum, that the dry matter is greater than 25 % of the whole digesta, the error due to this factor is not large enough to alter significantly the result. In addition, dilution was greater than five times in the large majority of cases and, as the error is halved for any given weight of digesta as the dilution passes from five to ten times, the maximum error due to the volume of dry matter cannot be greater than + 5 %.

A second source of error is due to sampling. When dealing with the rumen of the sheep or the ox or with the large intestine of the horse, the mass of material to be sampled is so great that the chances of obtaining representative samples seemed remote. Comparison of results obtained from duplicate samples, however, was encouraging. The percentage deviations from the mean of duplicate samples from digesta taken throughout the alimentary tract of two horses and two oxen are given in Table 1, and it will be seen that the maximum deviation occurs in the stomach and small intestine of the horse, regions in which little volatile acid is present, and consequently the error of titration is magnified. Otherwise the results are satisfactory, considering the bulk of the material and the comparatively crude method of obtaining the extract for distillation. The average percentage deviation for the two horses is 5·9 and for the two oxen 2·7.

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* We are greatly indebted to Dr J. A. Burkhardt for obtaining this data.

INTERPRETATION OF RESULTS

An inquiry into the meaning of the expression 'volatile acid', beyond the general statement made previously, is necessary. Many organic acids, other than fatty acids, are volatile in steam, but to a lesser extent than the fatty acids. In material, such as the digesta of the rumen or of the large intestine, other acids are present, besides acetic, propionic and butyric, and even if they are present in small quantities some may appear in the distillate.

Table 1. *Percentage deviation from the mean for duplicate samples*

	Horse 1	Horse 2
Stomach	7·7	14·8
Small intestine 1	13·4	12·0
Small intestine 2	7·7	6·4
Caecum	1·2	1·1
Colon 1	4·1	1·4
Colon 2	3·0	2·7
Rectum	2·0	4·6
	Ox 1	Ox 2
Rumen and reticulum:		
Dorsal sac	0·9	3·4
Ventral sac	2·7	1·8
Omasum	1·3	2·6
Abomasum	1·8	1·8
Small intestine 1	0·8	8·5
Small intestine 2	2·1	0·8
Caecum	5·2	7·3
Colon 1	1·9	3·2
Colon 2	2·5	1·6
Rectum	1·9	1·3

In order to determine what proportion of the acids, designated 'volatile acid', is represented by fatty acid, with the exception of formic acid, a series of analyses were done on digesta from a sheep and from a horse, in which the steam distillates were collected after titration and redistilled in the presence of acid mercuric sulphate solution and magnesium sulphate, according to the method of Friedemann (1938). This procedure removes formic acid, in addition to lactic and other non-fatty acids, and the remaining acids that are collected are fatty acids.

It will be seen from Table 2 that in the rumen of the sheep and in the large intestines of both animals over 85 % of the volatile acid is fatty acid, but in the small intestine the proportion of other acids becomes significant. This result was expected, for steam distillation of mixtures of the lower fatty acids, excluding formic acid, is complete when 150 ml. of distillate have been collected, and whereas distillation of extracts from the stomach of the ruminant or the large intestine of any animal was complete when this quantity of distillate had been collected, this was not so with extracts from the small intestine; with the latter, distillation of acids, other than acetic, propionic and butyric, was not complete when 150 ml. had been collected and small quantities of acid appeared in subsequent 50 ml. portions,

Table 2. *Fatty acid as percentage of total volatile acid*

A. Sheep	Total volatile acid as grams acetic acid		
	(*a*) By steam distillation	(*b*) By Friedemann distillation	(*b*) As percentage of (*a*)
Rumen and reticulum	86·5	75·1	87
Omasum	0·69	0·58	84
Abomasum	0·78	0·63	81
Small intestine 1	0·25	0·18	71
Small intestine 2	0·84	0·56	66
Caecum	6·15	5·92	88
Colon 1	2·17	1·89	87
Colon 2	0·28	0·24	86
Rectum	1·72	1·52	88
B. Horse	Grams acetic acid per 100 g. dry matter		
	(*a*) By steam distillation	(*b*) By Friedemann distillation	(*b*) As percentage of (*a*)
Stomach	1·09	0·83	76
Small intestine 1	0·50	0·35	72
Small intestine 2	1·13	0·63	56
Caecum	4·94	4·34	86
Colon	2·05	1·79	88

which infers that significant amounts of these other acids were present in the first 150 ml., the fraction taken in these analyses.

An alternative method

The method used, although simple and suitable for the work involved, is relatively crude; in order to investigate further its accuracy, the volatile acids of the digesta were extracted with ether to see if results obtained by this method agreed with those obtained by the usual aqueous method.

Extraction of the dried material with ether in a Sohxlet apparatus gave higher results than those obtained by aqueous extraction; on further examination this was found to be due to (1) the production of volatile acid during the process of drying the digesta under alkaline conditions, and (2) the possible production of volatile acid in the process of extraction, for extraction of digesta, from which the volatile acid had been recovered by repeated washing, gave a small but positive result.

Table 3. *Comparison of aqueous extraction with wet ethereal extraction*

	ml. of 0·1 N acid per 100 g. fresh rumen digesta		
	Sample 1 Aqueous extract	Sample 2 Aqueous extract	Sample 3 Wet ether extract
Exp. 1:			
By steam	96·0	100·0	106·0
By Friedemann	96·0	—	98·0
Exp. 2:			
By steam	25·5	23·2	27·1
By Friedemann	24·2	21·3	24·3
Exp. 3:			
By steam	80·8	83·2	79·8
By Friedemann	72·1	69·7	72·8

Extraction of dry matter with ether was abandoned in favour of ethereal extraction of wet digesta, using a Kutscher Steudel liquid extractor. By this method, results, very similar to those of aqueous extraction, were obtained after both extracts had been submitted to the Friedemann distillation.

It will be seen from Table 3, that, after steam distillation, the results of ethereal extraction were slightly higher than those obtained by aqueous extraction in two cases, but that this discrepancy became insignificant after the Friedemann distillation. The fact that the same result can be obtained by two different methods increases confidence in the methods employed in this work.

RESULTS

Foods

It is unlikely that the common foodstuffs fed on the farm, with the exception of silage, contain significant amounts of volatile acids; but it is important to know that the volatile acid found in the material in the stomach is not ingested with the food.

Aqueous extracts of minced foodstuffs were made with boiling water, and aliquots of the liquor obtained were treated in the same way as liquor obtained from digesta. The quantities of volatile acid found are insignificant, and when expressed as grams acetic acid per 100 g. dry matter gave the concentrations shown in Table 4.

When these concentrations are compared with those found in the rumen or with those found in the large intestine, it is obvious that volatile acid in these organs is produced *in vivo* and is not ingested with the food.

Ruminants

The characteristic feature of the results obtained from the ruminant is the large quantity of volatile acid present in the rumen and reticulum. In the sheep, this comprises 88 % of the total volatile acid found in the whole digesta of the alimentary tract; in the ox and the red deer the proportion is 86 and 90 % respectively.

The abrupt fall in the concentration of volatile acid between the rumen and the abomasum is an indication that the greater part of the volatile acid is removed from the digesta in transit; this is further accentuated by the fact that the percentage dry matter in the digesta of the rumen is in all cases, except one, higher than that of the digesta of the abomasum. It is known that absorption of volatile acids does occur in the omasum (Barcroft *et al*, 1944), and these facts imply that the omasum removes the greater part of the volatile acid from the digesta

Table 4. *Volatile acid content of foodstuffs*

Foodstuff	Grams acetic acid per 100 g. dry matter
Hay	0·12
Mangolds	0·33
Bran and oats	0·07
Grass	0·26

before they reach the abomasum. The percentage dry matter of the digesta in the omasum is higher than that of the digesta in the rumen; this is particularly marked in sheep, which suggests that either the digesta are compressed so that the liquid is squeezed from it into the abomasum or else that water is absorbed. The laminae of the omasum present a large absorbing surface, and it is known that water can pass from the blood into the rumen when that organ is filled with a hypertonic solution (Danielli, Hitchcock, Marshall & Phillipson, 1945). Presumably, the question of whether or not absorption of water takes place in the omasum depends upon the tonicity of the fluid digesta compared to that of the plasma.

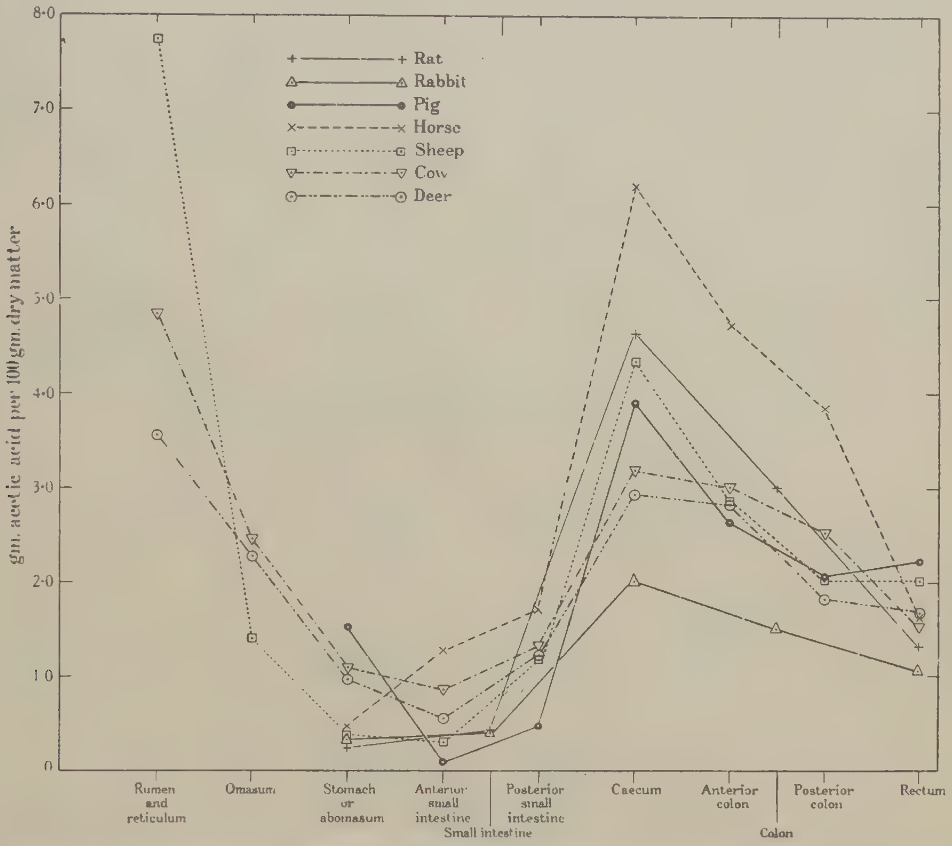

Fig. 1. The average concentrations of volatile acid, expressed as grams of acetic acid per 100 g. of dry matter, in relation to the part of the alimentary tract in which they were found.

The average results obtained from sheep, oxen and the red deer, for total volatile acid and volatile acid per 100 g. dry matter, are given in Table 5. The full results, including all essential data for individual animals, are given in tables at the end of the paper, and Fig. 1.

The quantity of volatile acid throughout the intestine is insignificant; and although the concentration rises abruptly in the caecum, the total quantity present is small, owing to the small amount of digesta contained in this organ. The large intestine in ruminants, therefore, as an organ in which fermentation occurs, does not compare with the rumen, although the rise in concentration of volatile acids shows that bacterial activity does occur there. The fall in concentration of volatile acids as the digesta pass towards the anus is accounted for by absorption as well as by concentration of dry matter, for the latter in itself is not sufficient to produce the decrease found.

Non-ruminants

In these animals the large gut was found to be the only part of the alimentary tract containing significant amounts of volatile acid. In the rat and rabbit, in which the colon is small compared to the caecum, the greatest quantity was found in the digesta contained in the latter organ. In the horse and pig, however, the colon is large compared to the caecum; consequently the greatest quantity was found in the colon, particularly the first portion which, by itself, contained more volatile acid than did the caecum. The quantity present in the stomach and small intestine is insignificant except in the pig, in which animal appreciable amounts are found in the digesta of the former organ. The reason for this is not clear, for the reactions of the digesta of the stomach of the horse, rat and pig probably vary between much the same limits, and structurally all three animals have similar organs with the exception of the oral half, which is lined by stratified epithelium in the horse and rat and by cardiac glands in the pig. The volatile acid in the food, fed prior to slaughter to each pig, was not responsible, since it was estimated in each case and found to be negligible.

Average figures for the total quantity of volatile acid in each organ in grams of acetic acid and for the concentration of volatile acid in grams of acetic acid per 100 g. dry matter are given in Table 6.

The concentrations throughout the large gut are similar in all cases; they are greatest in the caecum and decline progressively as the digesta pass on to the rectum. As in the ruminant, dehydration is insufficient to account for this fall.

Table 5. *Volatile acid in the digesta of ruminants. Grams of acetic acid*

	Sheep		Oxen		Red deer	
	Total	Concentration per 100 g. dry matter	Total	Concentration per 100 g. dry matter	Total	Concentration per 100 g. dry matter
Rumen and reticulum	63·6	7·7	329·0	4·8	65·2	3·55
Omasum	0·5	1·4	33·5	2·4	0·4	2·3
Abomasum	0·3	0·4	1·7	1·1	0·25	1·0
Small intestine 1	0·1	0·3	3·1	0·8	0·3	0·55
Small intestine 2	0·6	1·2	1·7	1·3	0·7	1·2
Caecum	4·6	4·3	5·7	3·2	2·7	2·9
Colon 1	1·1	2·85	1·3	3·0	1·1	2·8
Colon 2	1·4	2·0	2·85	2·5	0·7	1·8
Rectum	1·0	2·0	2·3	1·5	1·7	1·7

Table 6. *Volatile acid in the digesta of the horse, pig, rabbit and rat. Total as grams of acetic acid; concentration as grams of acetic acid per 100 g. of dry matter*

	Horse		Pig		Rabbit		Rat	
	Total	Concentration	Total	Concentration	Total	Concentration	Total	Concentration
Stomach	4·0	0·45	2·44	1·52	0·02	0·3	0·001	0·2
Small intestine 1	2·0	1·3	0·01	0·08	0·01	0·4	0·001	0·4
Small intestine 2	5·7	1·7	0·27	0·47				
Caecum	45·5	6·2	1·51	3·91	0·34	2·0	0·015	4·6
Colon 1	136·0	4·7	2·67	2·64	0·02	1·5	0·002	3·0
Colon 2	54·1	3·85	0·92	2·06				
Rectum	13·8	1·6	0·63	2·22	0·02	1·1	0·005	1·3

Table 7. *Volatile acid in the large intestine of individual horses*

	Total weight of digesta in caecum and colon in g.	Total volatile acid in caecum and colon in g. acetic acid	Ratio of digesta to volatile acid	Volatile acid as g. acetic acid per 100 g. dry matter
Horse 1	65,670	248·6	261 : 1	3·87
Horse 2	58,067	355·45	163 : 1	4·95
Horse 3	19,850	103·8	191 : 1	2·58
Horse 4	74,150	331·0	224 : 1	—

The total quantity present in the caecum and colon of the horse calls for comment; the total figures for the three animals examined for these organs are 248·6, 355·45 and 103·8 g. respectively and they show an apparently wide deviation, but the animals from which these results were obtained varied considerably in weight. A comparison of the volatile acid content of the digesta of the large intestine to the total weight of the digesta is given in

Table 7, which included an additional horse in which the volatile acid was estimated in the large gut only and is not included in the averages given in the previous table. The ratio between the whole digesta and the total volatile acid, although varying between the limits of 1 : 163 and 1 : 261, places the results on a more comprehensive basis; the large intestine of horse 3, which contains least volatile acid, proportionately contains more than the large intestines of horses 1 and 4.

Table 8. *Volatile acid in the large intestine of individual pigs, rats and rabbits*

	Total weight of digesta in caecum and colon in g.	Total volatile acid in caecum and colon in g. acetic acid	Ratio of digesta to volatile acid
Pig 1	1298·0	7·98	163 : 1
Pig 2	1396·0	8·72	160 : 1
Pig 3	250·0	1·43	175 : 1
Pig 4	468·0	2·28	205 : 1
Rats 1–5	19·85	0·108	183 : 1
Rats 6–11	15·19	0·094	162 : 1
Rats 12–17	14·05	0·090	156 : 1
Rabbit 1	65·3	0·21	315 : 1
Rabbit 2	70·5	0·25	286 : 1
Rabbit 3	135·0	0·67	200 : 1
Rabbit 4	130·5	0·22	604 : 1
Rabbit 5	98·6	0·46	217 : 1

Table 9. *Partition of volatile fatty acid*

Animal	Organ	% of fatty acids		
		Acetic	Propionic	Butyric
Ox	Rumen*	65·8	21·2	13·0
	Rumen*	69·6	16·5	13·9
	Rumen*	71·3	14·1	14·6
	Rumen	64·0	21·3	14·7
	Caecum	70·8	17·1	12·1
	Caecum	72·9	18·4	8·7
Red deer	Rumen	71·2	14·9	13·9
Horse	Caecum	72·0	22·5	5·5
	Caecum	74·3	16·8	8·9
	Colon	67·0	19·5	13·5
Pig	Caecum	62·1	27·8	10·1
Rabbit	Caecum	74·8	8·0	17·2
	Caecum	81·7	10·6	7·7
Rat:				
5 animals	Caecum	56·1	30·8	13·1
6 animals	Caecum	40·2	17·8	42·0

* Additional material obtained from slaughterhouse.

If the results for the individual animals in the remaining species are compared in a similar way in Table 8, the pig and the rat, on this basis, are comparable to the horse, while the rabbit tends towards a wider ratio.

The individual acids found in the digesta

It was stated earlier that 85 % of the volatile acid present in the rumen or in the large intestine consisted of fatty acid other than formic. The fact that the mixture of fatty acids, present in the rumen, consists of acetic, propionic and butyric, has been demonstrated by Elsden (1945) by means of chromatographic partition. This method has been applied to distillates, obtained from animals examined here, with similar results to those for the rumen. The average results obtained are given in Table 9.

In all cases acetic acid predominates and the figures show remarkable agreement for all species. This is even more noteworthy when it is recalled that the diets varied from heather in the case of the deer to bran in the case of the pig. One exception to the general rule is the rat; for in the first group of five animals the proportion of acetic acid is low and that of propionic acid high compared to the other animals, while in the second group of rats the proportion of both acetic and butyric acids is approximately 40%. This is the only instance in which a high proportion of butyric acid was found.

The similarity of the results from seven different species, representing four separate orders, is sufficient evidence upon which to make the statement that fermentation within the alimentary tract is characterized by the production of acetic, propionic and butyric acids and that acetic acid predominates in the majority of cases, the proportion of this acid being approximately 67 %—the average proportion obtained from the figures given in the table.

Quantitative extraction of volatile acids from the large intestine of the horse

The isolation of volatile acid from the digesta of the large intestine of the horse was undertaken in order to give final proof that the quantities estimated were in fact present. The total weight of digesta in the caecum and colon used was 74·15 kg. The material was taken in portions, mixed with water, and the liquor strained off through sacking into carboys. Each portion was treated in this way at least three times until finally six carboys of liquor, containing in all 272 l., were obtained. This liquor was evaporated in a large bath, the *p*H being maintained between 8 and 9, until a thick tar-like residue was obtained. This was transferred in portions into a large porcelain dish and dried over a steam bath and finally in a steam oven. The material thus obtained was broken into small pieces and ground to a fine powder in a hammer mill. The weight of the powder obtained was 2905 g.

The powder was divided into four parts, and each, taken in turn, was mixed with anhydrous acid potassium sulphate, moistened with a minimum quantity of water, placed in capsules in a large Sohxlet apparatus* and extracted with ether. The ether containing the extracted material was distilled off over alkali so that the volatile acid on neutralization passed into the aqueous phase. The volatile acid was subsequently distilled from the aqueous solution after acidification and the distillates were titrated with 40 % KOH. The total acid obtained in this way amounted to 342·5 g. expressed as acetic acid. The mixed acids were redistilled and water was removed by evaporation after the acid was neutralized, leaving a white crystalline powder which was finally dried in a desiccator.

It was calculated from samples taken from various parts of the digesta of the caecum and colon and treated in the usual way, that the total quantity of volatile acid present in the digesta was 331 g., expressed as acetic acid. The quantity actually extracted was 342·5 g., a figure higher than the calculated amount by 3·4 % and not outside experimental error.

The statement was made earlier in this paper that drying rumen contents under alkaline conditions resulted in a significant increase in the amount of volatile acid present as shown by distillation of aqueous extracts of dry matter. This increase became greater as the alkalinity rose. The extraction of the total amount of volatile acid from digesta of the caecum and colon of the horse described here was done on an aqueous extract of the digesta and not on the whole digesta as was the case with rumen contents. Drying the liquor of rumen digesta under alkaline conditions does not give rise to additional volatile acid to any significant extent, and samples treated in this way have increased by 8 % at the most. It is considered, therefore, that the procedure adopted with the digesta of the horse described here would not introduce an error greater than this.

DISCUSSION

The amount of volatile acid present in the digesta depends upon the rate at which it is produced and the rate at which it disappears from any one organ. Beyond the fact that the concentration of volatile acid in the liquid contents of the rumen can be doubled in a few hours of feeding (Phillipson, 1942), little can be said about the rate at which volatile acid is produced. Volatile acid in the digesta of any organ may disappear by three routes: it may (1) pass on with the digesta to the succeeding organ; (2) it may be directly absorbed into the blood stream; and (3) it may be utilized by bacteria or other micro-organisms as a source of energy, in which case volatile acids cannot be considered as the end-product of fermentation in the alimentary tract.

If volatile acid leaves any organ, with the digesta, quantitatively, then the concentration in the adjacent organ, assuming no more is produced there, could be calculated from the variation in dry matter between the two organs. In no case is this so. The increase in dry matter as the digesta pass from the rumen to the omasum and as the digesta pass from the caecum to the rectum, is insufficient to produce the fall in concentration of volatile acid that occurs. Additional volatile acid must therefore be lost by either or both routes 2 and 3.

There is direct evidence that volatile acid is absorbed from the organs in which it is produced, for its presence has been demonstrated in the blood leaving these organs (Barcroft *et al.* 1944). It is possible that different rates of absorption may account for the different amounts of acid present, and that, when the amount of digesta is proportionately small, absorption is more rapid, so that the concentration present at any time is proportionately less than that present in organs containing proportionately more digesta.

It is possible that, in addition to absorption, some volatile acid is further broken down by bacteria for their own use. There is no positive evidence to show that this occurs to any significant extent in the rumen; indeed, the fact that such large quantities accumulate there suggests that it does not. The rapidity with which lactic acid, which is known to be an intermediary product of fermentation in the rumen, disappears both *in vivo* and *in vitro* (Phillipson & McAnally, 1942) supports the view that acetic, propionic and butyric acids are not utilized by bacteria in the rumen either rapidly or to any great extent.

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* The Sohxlet apparatus was kindly lent to us by Prof. A. C. Chibnall, F.R.S.

The figures given in Table 10, showing the proportion of live weight due to digesta, together with the proportion of volatile acid to live weight, suggest that, taken on a broad basis, the amount of volatile acid present depends upon the total quantity of digesta. If, however, the proportion of live weight that is due to the digesta is assumed to be 25·3 % in all species, as it is in the ox, and the figures for the volatile acid per kg. body weight are multiplied by the appropriate factor, as in Table 11, then the amount of volatile acid present in the digesta is greater in the ox and the sheep than in those animals in which fermentation occurs principally in the large intestine. Within the latter group, the proportion of volatile acid present is much the same except for the rabbit, in which it is smaller. Owing to the small numbers of animals used, conclusions based upon average figures can only be tentative; on all counts, however, the quantity of volatile acid in the ox is greater than that in any other animal, and the sheep, when compared on the same basis, is its equal; the difference between these animals and the rabbit at the other extreme can be due either to greater fermentation in the ox and sheep or to greater absorption in the rabbit. It is not possible, therefore, until more is known regarding the absorption of volatile acid from the large intestine, to consider figures for concentration or total amount of volatile acid in any organ as an infallible measure of the amount of fermentation, except where individuals of one species are concerned. In considering the values obtained for horses, whichever figure for the live weight is taken, the result is much the same, and therefore it is considered that the values given are comparable to those for the other animals in which the live weight was obtained by direct weighing.

Table 10. *Comparison of proportion of volatile acid and digesta to live weight*

	Digesta as % of body weight	Volatile acid as % of digesta	Grams of volatile acid per kg. body weight
Ox	25·3	0·58	1·50
Sheep	12·6	0·67	0·76
Horse: (*a*)	12·5	0·39	0·48
(*b*)	17·1	0·39	0·67
Pig	11·4	0·40	0·40
Rabbit	17·7	0·18	0·33
Rat	3·75	0·33	0·12

(*a*) Taken from calculated live weight. (*b*) Taken from slaughterman's estimate of live weight.

Table 11. *Proportion of volatile acid to live weight after adjustment of proportion of digesta to a constant*

		Grams of volatile acid per kg. body weight
Ox	—	1·50
Sheep	0·76 × 25·3/12·6	1·52
Horse: (*a*)	0·48 × 25·3/12·5	0·97
(*b*)	0·67 × 25·3/17·1	0·99
Pig	0·40 × 25·3/11·4	0·89
Rabbit	0·33 × 25·3/17·7	0·47
Rat	0·12 × 25·3/3·75	0·81

CONCLUSIONS

1. A mixture of acetic, propionic and butyric acids is a characteristic product of fermentation in the alimentary tract of sheep, ox, red deer, horse, pig, rabbit and rat. The relative proportions of these acids are very similar in all species with the exception of the rat, and are on an average 67 of acetic acid to 19 of propionic acid and 14 of butyric acid.

2. Over 85 % of the steam-distilled volatile acids of the rumen and large intestine consists of acetic, propionic and butyric acids.

3. The regions of the alimentary tract, in which fermentation occurs, are sharply defined and consist of rumen and reticulum in ruminants and the large intestine of all seven species.

4. The proportion of volatile acid per kg. body weight is highest in the ox and sheep; it progressively declines from the horse, pig and rabbit to the rat.

5. The average quantities of volatile acid computed as being in the rumen and reticulum of the ox are 329 g., in the sheep 64 g. and in the red deer 65 g.; in the colon and caecum of the horse 236 g. and in the young pig 5·6 g. 342·5 g. of mixed volatile acid (expressed as acetic acid) have actually been isolated from the large intestine of a horse.

The authors are greatly indebted to Sir Joseph Barcroft, F.R.S., for his interest in this work. It was at his instigation that it was extended to other animals besides the sheep, while the extraction of volatile acid from the digesta of the large intestine of the horse was prompted by the incredulity expressed by certain members of the Agricultural Research Council when they were informed by him of the quantities calculated to be present.

Table 12. *Sheep*

Sheep 1: 83 kg. live wt. Diet: grass. Sheep 2: 86 kg. live wt. Diet: grass. Sheep 3: 77 kg. live wt. Diet: grass. Sheep 4: Diet: hay and a concentrated mixture of bran, oats, cracked beans and linseed cake. Sheep 5: Rumen only: 6850 g. wt. ingesta, 9·7 % dry matter, 64·76 g. total volatile acid; 9·74 g. volatile acid per 100 g. dry matter.

	g. wt. ingesta				% dry matter			
Sheep no. ...	1	2	3	4	1	2	3	4
Rumen and reticulum	5750	8650	7250	8400	5·80	11·30	11·35	19·40
Omasum	130	30	95	30	24·80	25·65	24·80	24·00
Abomasum	460	820	510	1099	9·20	7·30	5·60	10·20
Small intestine 1	415	294	433	412	12·70	9·70	8·70	12·20
Small intestine 2		838	530	464		9·40	9·50	10·10
Caecum	605	1013	628	748	15·60	12·80	12·60	15·20
Colon 1	139	160	217	283	20·30	20·00	14·90	17·10
Colon 2	90	66	132	68	26·30	22·30	16·40	21·90
Rectum	90	133	314	287	28·10	22·90	19·10	24·80

	Total volatile acid g. acetic				g. volatile acid per 100 g. dry matter			
Sheep no. ...	1	2	3	4	1	2	3	4
Rumen and reticulum	26·80	74·90	65·29	86·50	8·10	7·64	7·93	5 29
Omasum	0·70	0·09	0·34	0·69	2·10	1·12	1·44	0·95
Abomasum	0·15	0·06	0·06	0·78	0·20	0·11	0·21	0·97
Small intestine 1	0·20	0·04	0·14	0·25	0·40	0·14	0·37	0·52
Small intestine 2		0·76	0·39	0·84		0·96	0·77	1·80
Caecum	3·80	5·55	2·86	6·15	4·00	4·28	3·60	5·41
Colon 1	0·50	0·67	0·93	2·17	1·90	2·11	2·88	4·49
Colon 2	0·50	0·23	0·57	0·28	2·10	1·57	2·64	1·85
Rectum	0·50	0·51	1·17	1·72	2·00	1·66	1 95	2·42

Table 13. *Oxen*

Steer 1: 2½ years; 364 kg. live wt. Steer 2: 1 year; 182 kg. live wt.

	g. wt. ingesta		% dry matter		Total volatile acid g. acetic		g. volatile acid per 100 g. dry matter	
Ox no. ...	1	2	1	2	1	2	1	2
Rumen and reticulum	54,090	36,820	13·30	17·25	371·20	286·40	5·13	4·51
Omasum	8,410	6,590	17·90	19·30	35·90	31·00	2·38	2·44
Abomasum	1,140	1,590	12·60	10·90	1·71	1·68	1·19	0·97
Small intestine 1	4,320	4,320	7·80	8·95	2·62	3·52	0·77	0·91
Small intestine 2	2,730	1,140	5 90	9·00	2·33	1·17	1·45	1·14
Caecum	2,500	1,590	7 20	11·00	5·97	5·34	3·30	3·05
Colon 1	230	680	7·40	11·00	0·52	2·17	3·08	2·90
Colon 2	910	910	8·20	11·90	2·01	2·50	2·69	2·31
Rectum	1,140	1,360	10·20	13·65	1·61	3·04	1·38	1·64

Table 14. *Red deer*

	g. wt. ingesta	% dry matter	Total volatile acid g. acetic	g. volatile acid per 100 g. dry matter
Rumen and reticulum	13,180	14·0	65·24	3·55
Omasum	110	16·8	0·41	2·26
Abomasum	220	12·1	0·25	0·96
Small intestine 1	450	10·5	0·26	0·55
Small intestine 2	910	6·3	0·70	1·22
Caecum	910	10·2	2·73	2·93
Colon 1	340	11·3	1·08	2·82
Colon 2	230	17·1	0·70	1·82
Rectum	450	22·2	1·66	1·66

Table 15. *Horses*

Horse 1: 662 kg. live wt. Horse 2: 458 kg. live wt. Horse 3: 159 kg. live wt.

	g. wt. ingesta			% dry matter		
Horse no. ...	1	2	3	1	2	3
Stomach	1,000	13,115	2,495	8·40	18·95	9·59
Small intestine 1	4,600	8,127	1,600	5·96	6·32	1·98
Small intestine 2	5,850	10,418	2,150	3·66	6·70	6·74
Caecum	11,750	15,859	3,350	4·42	5·85	9·67
Colon 1	35,550	24,827	10,450	11·48	11·07	13·41
Colon 2	18,450	17,381	5,050	9·74	10·54	12·21
Rectum	6,850	8,045	2,800	17·48	13·54	16·01

	Total volatile acid g. acetic			g. volatile acid per 100 g. dry matter		
Horse no. ...	1	2	3	1	2	3
Stomach	0·13	9·90	1·94	0·15	0·40	0·81
Small intestine 1	0·64	4·73	0·74	0·23	1·23	2·34
Small intestine 2	1·33	11·89	3·91	0·62	1·76	2·70
Caecum	28·60	100·85	7·16	5·51	10·88	2·21
Colon 1	166·10	169·90	72·88	4·07	6·18	4·06
Colon 2	53·90	84·70	23·72	3·00	4·62	3·92
Rectum	6·40	25·66	9·24	0·53	2·36	2·00

Table 16. *Pigs*

Pig 1: weight not known. Pig 2: 21·7 kg. live wt. Pig 3: 3 months. Live wt. not known. Pig 4: 19·1 kg. live wt.

	g. wt. ingesta				% dry matter			
Pig no. ...	1	2	3	4	1	2	3	4
Stomach	842	647	443	811	22·1	23·3	25·9	23·4
Small intestine 1	118	76	270	350	9·9	13·9	9·0	12·4
Small intestine 2	522	350			12·5	14·6		
Caecum	239	424	64	242	13·0	15·9	21·5	13·1
Colon 1	740	712	94	180	16·9	19·1	27·6	22·0
Colon 2	319	260	92	46	20·4	21·3	27·1	27·6
Rectum	189	159	32	15	20·8	21·1	28·6	23·3

Table 16 (continued)

	Total volatile acid g. acetic				g. volatile acid per 100 g. dry matter			
Pig no. ...	1	2	3	4	1	2	3	4
Stomach	3·20	2·72	1·11	2·72	1·73	1·81	1·09	1·43
Small intestine 1	0·02	0·00	0·25	0·31	0·16	0·00	1·02	0·71
Small intestine 2	0·26	0·27			0·40	0·53		
Caecum	1·60	2·81	0·28	1·36	5·16	4·17	2·02	4·27
Colon 1	4·57	4·68	0·66	0·76	2·66	3·44	2·55	1·92
Colon 2	1·80	1·23	0·49	0·16	2·77	2·45	1·98	1·25
Rectum	0·99	0·09	0·08	—	2·54	2·45	1·67	—

Table 17. *Rabbits*

Rabbit 1: 1055 g. live wt. Rabbit 2: 1017 g. live wt. Rabbit 3: 1242 g. live wt. Rabbit 4: 1047 g. live wt. Rabbit 5: 1490 g. live wt. Rabbit 6: Caecum only: 109·2 g. wt. ingesta, 27·1% dry matter, 0·356 total volatile acid as acetic, 1·42 g. volatile acid per 100 g. dry matter. Rabbit 7: Caecum only: 83·5 g. wt. ingesta, 23·0 % dry matter, 0·296 total volatile acid as acetic, 2·14 g. volatile acid per 100 g. dry matter.

	g. wt. ingesta					% dry matter				
Rabbit no. ...	1	2	3	4	5	1	2	3	4	5
Stomach	99·0	84·0	92·0	38·5	82·2	23·7	20·9	10·0	9·1	18·8
Small intestine 1	12·0	15·0	12·5	10·0	31·8	8·5	6·0	8·0	7·7	9·7
Small intestine 2			11·0	14·0				5·5	8·2	
Caecum	59·0	60·0	118·5	120·5	94·7	19·6	28·8	12·4	17·9	20·7
Colon	6·3	10·0	16·5	10·0	3·9	34·5	7·5	12·8	16·5	28·2
Rectum	7·0	10·5	3·5	4·0	6·0	46·0	12·3	23·5	34·4	30·3

	Total volatile acid g. acetic					g. volatile acid per 100 g. dry matter				
Rabbit no. ...	1	2	3	4	5	1	2	3	4	5
Stomach	0·008	0·019	0·027	0·003	0·050	0·03	0·11	0·29	0·86	0·32
Small intestine 1	0·001	0·000	0·011	0·002	0·013	0·10	0·00	1·10	0·27	0·41
Small intestine 2			0·004	0·003				0·66	0·26	
Caecum	0·204	0·216	0·628	0·203	0·439	1·76	1·25	4·27	0·94	2·24
Colon	0·003	0·030	0·046	0·013	0·016	0·16	3·05	2·09	0·79	1·47
Rectum	0·007	0·030	0·006	0·003	0·033	0·22	2·32	0·73	0·22	1·82

Table 18. *Rats*

Batch 1: 5 rats; wt. not recorded. Batch 2: 6 rats; 1268 g. live wt. Batch 3: 6 rats; 963 g. live wt.

	g. wt. ingesta			% dry matter			Total volatile acid as mg. acetic			g. volatile acid per 100 g. dry matter		
Batch no. ...	1	2	3	1	2	3	1	2	3	1	2	3
Stomach	6·03	8·33	11·47	28·7	38·6	32·3	5·0	4·4	9·8	0·29	0·14	0·26
Small intestine	7·76	8·46	11·76	14·5	12·2	14·5	6·9	3·0	6·5	0·61	0·29	0·38
Caecum	16·81	12·81	10·80	14·9	12·2	13·1	99·0	79·8	72·0	3·94	5·11	4·87
Colon	3·04	2·38	3·25	16·3	15·3	16·9	9·6	13·7	17·6	1·91	3·80	3·20
Rectum	7·75	6·51	6·45	32·5	31·0	38·7	20·5	31·0	29·4	0·81	1·53	1·60

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THE INFLUENCE OF PERIPHERAL CONNEXION ON THE DIAMETER OF REGENERATING NERVE FIBRES

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(With Plates 4–7 and Four Text-figures)

INTRODUCTION

Nerve fibres subserving different functions have different diameters, not only in a normal nerve but also in one which has been crushed and has undergone degeneration and regeneration (Gutmann & Sanders, 1943). Therefore, there must be factors operating during the regeneration of any particular nerve which tend to restore the fibre size pattern typical of that nerve.

Simpson & Young (1945) have shown by cross union of visceral and somatic nerves containing fibres of different diameters that the central stump exerts an influence in determining the size of fibres regenerated. Thus after union of the central stump of a somatic nerve with the postganglionic trunk of the anterior mesenteric nerve, the latter comes to contain many medullated fibres, whereas normally this nerve contains very few medullated fibres in the rabbit.

However, there is also evidence of a strong peripheral influence upon the size attained by regenerating fibres. When a mixed nerve is severed and its ends united, there is such confusion of fibres at the line of suture that if central influences alone controlled the diameter of regenerated fibres, then all branches of the nerve below the suture would be expected to regenerate fibres of similar sizes. This in fact is not the case. Nageotte & Guyon (1918) and Sanders & Young (1944) have shown that below a suture in a mixed nerve the muscular branches come to contain larger fibres than the cutaneous. After degeneration the cutaneous branches contain smaller Schwann tubes, and it has been suggested that it might be the restrictive influence of these which produced the difference (Gutmann & Sanders, 1943; Sanders & Young, 1944). Such a restrictive influence operates to some extent, but further experiments have shown that the most powerful influence lies not in the peripheral stump itself, but in connexion with the end-organ (Sanders & Young, 1945; Simpson & Young, 1945). Fibres which become connected with a muscle become larger than those which end blindly. Weiss & Taylor (1944) found evidence of a similar effect in the course of experiments in which they completed the disproof of the theory of 'neurotropism'. More recently, Weiss, Edds & Cavanaugh (1945) have extended this work and shown in various nerves of rats that a stretch of nerve regenerating without opportunity for its fibres to reach the periphery becomes filled with many and small nerve fibres.

The present work contains an investigation of the numbers and sizes of fibres found in nerves regenerating with and without normal peripheral connexions. Since fibres with no peripheral connexions are shorter than those regenerating from the same level all the way to the periphery, a control investigation has been made of the effect of length of nerve on the diameter reached during regeneration. Finally, in order to discover whether the influence of peripheral connexion can override that of size of peripheral Schwann tubes, we have also made a study of regeneration through nerve grafts which originally contained fibres of different sizes. This serves also to answer a question of considerable practical importance to the surgeon who wishes to use pieces of cutaneous nerve for grafting into defects in motor nerve trunks (see Sanders & Young, 1944; Hammond & Hinsey, 1945).

METHODS

The difficulty of this, as of many other investigations of nervous regeneration, has been to obtain similar lesions of the nerves in successive experiments. If two nerves are cut and reunited in such a way that the interval between the ends differs, then the size of the fibres regenerated in the two cases will also differ (Simpson & Young, 1945; Gutmann & Sanders, 1943). We have overcome this difficulty by interrupting the axons by crushing them with narrow smooth-tipped watchmaker's forceps. If carefully performed, this operation interrupts the axons but leaves intact the connective tissue tubes in which they run, thus leaving optimal and standard opportunities for regeneration.

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The peroneal nerves of rabbits were crushed in this way in the thigh, and then severed lower down, the ends being so treated as to provide different conditions for the reception of the regenerating fibres. The details of the various experiments are given below. All unions of the nerves were made by means of concentrated cockerel plasma, for the supply of which we are indebted to Mr P. B. Medawar.

For measurement of the regenerating fibres the nerves were fixed in Flemming's fluid,* sectioned transversely at 5μ after paraffin embedding, and stained with a modified Weigert method (Gutmann & Sanders, 1943). This did not always prove satisfactory, since the fixative in some cases failed to penetrate adequately to the centre of the nerve. The fibres were counted and measured on negative photographs made by direct projection on bromide paper at a magnification of 750 × . In some cases the whole nerve was photographed, while in others sample strips across the nerve were used. After photographing, a number of sample areas were selected for counting by marking out squares 50 × 50 mm. on the finished photograph, usually in a ring round the periphery of the nerve, where fixation, by ordinary histological criteria, was deemed to be adequate. The area of the whole nerve was also determined in each case by planimetry either of the photograph of the whole nerve or of a projection. The fibres in each sample square were then counted and marked off in size-groups with an interval of 2μ. The fibres of various sizes are not scattered quite at random throughout the nerve but sufficient squares were taken to provide an adequate total sample of the nerve. Except in special cases, mentioned elsewhere in the text, 25–30 squares were measured in the case of each nerve, an area equivalent to rather more than a quarter of the total area of the nerve. From the total number of fibres in the sample measured and the total nerve area obtained by planimetry an estimate of the total population could be obtained. The errors involved in such counts are discussed by Gutmann & Sanders (1943) and Simpson & Young (1945). In the present experiments nerves of the opposite sides of individual animals were compared, and since the technique was kept constant, errors of absolute estimation were unimportant.

Method of expressing results

In quantitative investigations of the present kind it is important to use a measure which shall express adequately the constitution of each individual nerve examined. The average fibre diameter is not very suitable for this purpose. Since the curve of distribution of fibre sizes departs quite widely from the form of the 'normal curve', being in many cases quite strongly bimodal, the average fibre diameter does not typify the diameter of the greatest number of fibres in the nerve. Moreover, there is such a numerical preponderance of small myelinated fibres in most nerves that nerves which differ strikingly in fibre constitution (e.g. muscular and cutaneous bundles) differ only slightly in average fibre diameter. Accordingly, we have in this paper used the root mean square of the whole population of diameters as our estimate of 'mean' fibre diameter. This figure (D) was calculated separately for each square of the sample counted, and the value of D given for each nerve was the mean of all the D's found for separate squares within that nerve. This method of calculating D has the advantage that an estimate of error can be made in each case, and the values of D obtained for different nerves compared statistically. This estimate of the 'mean' diameter has an additional advantage in that it is directly related to the total volume of nerve fibres present in the nerve. If the nerve were wholly composed of fibres of diameter D it would have a volume of fibres per unit length equal to that actually found in the nerve, but there spread over fibres of various diameters.

RESULTS

Effect of length on diameter of regenerated fibres

The object of the experiments described in this section was to discover whether the diameter reached by the fibres at any level in a regenerating nerve is affected by the length of fibre regenerating peripheral to that level. Extension of a nerve beyond its normal length can be achieved either by the use of a graft or by joining it with another nerve. Both methods have considerable disadvantages. The difficulty of obtaining suitable lengths of nerve for autografting and the inevitable variability in the resulting junctions suggested that the method of cross union might be more suitable. The peroneal nerves were therefore crushed as high as possible on both sides of each animal, cut lower down, and joined to the tibial nerves in such a way as to make on one side a nerve of normal length, and on the other one in which fibres have to grow much farther than is usual.

This was done by freeing the peroneal nerve along its whole length, cutting it peripherally, turning it back along itself and joining it to the tibial high in the thigh. The fibres regenerating from the crushed points thus have to pass through a much shorter length of peroneal nerve on the one side before entering the tibial and passing to the end-organs of the latter.

This procedure has the disadvantage that none of the fibres reaches its normal end-organ. However, both the tibial and the peroneal are nerves which have both muscular and cutaneous destinations, the tibial being the larger. It is known that the peroneal motor fibres which enter the gastrocnemius muscles make endings such that stimulation of the nerve causes contraction of these muscles, but it is uncertain to what extent such abnormal connexions function during life. In any case, the final terminations are equally abnormal on both sides of the animal, and the only difference is therefore in the length of nerve to be regenerated.

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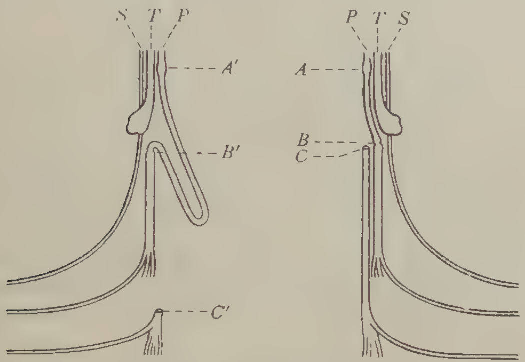
* 1% chromic acid in distilled water, 15 c.c., 2% osmic acid, 4 c.c., glacial acetic acid, 2 drops.

Two experiments of this kind were made, rabbit 1011 being killed 107 days, and rabbit 1010, 200 days after operation.*



Text-fig. 1. Diagrams of operations performed in order to increase the length of fibres regenerated in the peroneal nerve. Peroneal nerve, *P*, is crushed high in the thigh (*A*, *A′*) and lower down severed and joined to the tibial nerve, *T*, at *B* or *B′*; *C* or *C′*, peripheral stumps of peroneal nerve; *S*, sural nerve.

107 *days of regeneration.* In 1011 the distance on the side with a short nerve (*AB*; see Text-fig. 1) between crushed point and the union with the tibial nerve was 20 mm., whereas *A′B′*, the distance from the crushed point to the union on the opposite side of the animal, was 75 mm. From the results of Gutmann, Guttmann, Medawar & Young (1942) we may assume that the tips of the regenerating axons on both sides cross the region of crush 5 days after the operation, and advance through the peroneal nerve at 4·4 mm./day. Conditions in the two nerves are the same, except that on the one side the fibres have to travel an extra 55 mm. We may, therefore, expect that the fibres will arrive in the muscles supplied by the long nerve 55/4·4 = 12·5 days later than on the short side. Out of a total regeneration time of 107 days, the fibres in the short nerve will have had 12·5 days longer for maturation after the arrival of fibres.

In order to discover what difference this increased length made to the process of maturation, sections were made of the peroneal nerves 10 mm. below the crushed point. Table 1 shows that the estimated total number of fibres is similar in the two nerves. *D*, the 'mean' fibre diameter (see p. 204), is slightly greater in the longer nerve, but since differences of this magnitude can be expected to occur in 60–70 % of cases as a result of the operation of chance factors, the difference cannot be termed significant. Therefore, neither the fact that a greater length of nerve was regenerated, nor that contact with the muscle was made later, had operated to affect the diameter of the fibres immediately below the crushed point (Pl. 4, figs. 1, 2).

Table 1. *The effect of length of nerve on fibre maturation*

Animal	Days	Level	Length of nerve below crush	No. of fibres	*D* in μ	Comparison		
						Degrees of freedom	*t*	*P*
1011 *g*	107	Central stump	Long	9817	7·4	63	1·691	0·5–0·10
1011 *b*	107	Central stump	Short	9412	·8·1			
1011 *i*	107	Peripheral stump	Long	6577	6·1	41	0·869	0·3–0·4
1011 *d*	107	Peripheral stump	Short	5539	6·0			
1010 *c*	200	Peripheral stump	Long	7326	6·9	34	3·519	0·001–0·01
1010 *m*	200	Peripheral stump	Short	7066	6·3			

Sections were also made farther down both nerves, and showed plentiful medullated fibres below the suture points on both sides (Pl. 4, figs. 3, 4; 1011 *f*, *k*). No large difference was evident in the fibre diameters but counts were not made. It seems that at this level two equally good maturation has been achieved on the sides in spite of their unequal distances from the lesion.

Still farther distally this is no longer true. Sections of the posterior tibial nerves above the heel showed only very few medullated fibres in the nerve which had been artificially lengthened (Pl. 4, fig. 5; 1011 *l*), whereas they were more abundant and larger on the other side (Pl. 4, fig. 6; 1011 *m*). On both sides the fibres were much smaller than at more proximal levels. Evidently the greater length to be regenerated had allowed less full maturation.

* Every piece of nerve removed from an animal is indicated by a small letter, thus 1011 *b* means piece *b* of rabbit 1011; see tables.

We were also interested to discover whether the length of new nerve fibres to be formed affected the diameter of fibres *central* to the lesion. Table 1 shows that *D* was in fact smaller in the nerve which had the longer distance to regenerate, the difference between the two sides being of a magnitude that would only be found in 5–10% of cases as a result of random sampling alone. 100 days after injury is the time at which the fibres of the central stump show their greatest decrease in volume (Gutmann & Sanders, 1943), and if this decrease is in some way connected with the outpouring of material to make the new fibres, it might be expected to be greater in the nerve which has the greater length to regenerate.

Sections of the points of union of the peroneal and tibial nerves showed good junctions on both sides (Pl. 4, figs. 7, 8; 1011 *e, j*) without any waist-like constriction. Although, unfortunately, there is no means of making a quantitative assessment of such junctions, we feel safe in concluding that these are similar on the two sides, and therefore that the absence of any difference in fibre diameter is significant.

200 *days of regeneration*. In rabbit 1010 the nerves were arranged so far as possible exactly as in 1011, and left to regenerate for 200 days. Using the same assumptions as before, we may conclude that the new fibres arrived at the gastrocnemius muscles 10 days later on the 'long' side, and therefore had that much longer for maturation in the short nerve.

Table 1 shows that 10 mm. below the point of crushing there were again approximately the same number of fibres on both sides. At this level, however, there were rather larger fibres on the side with the longer nerve. Moreover, this difference is greater than any to be expected as a result of random sampling. It will be noticed that the fibres are larger than after 100 days, though not greatly so (Pl. 5, figs. 9, 10).

Below the points of suture of the peroneal to the tibial nerves no actual counts were made, but the fibres appear smaller on the side with the longer nerve. On both sides there is a distinct difference between levels above and below the suture in that, whereas at the former there is usually only one large fibre per tube, at the latter there are several smaller ones (Pl. 5, figs. 9–12; 1010 *c,m,g,q*). This is a distinction regularly observed between nerves regenerated below crushed and sutured points. In this case the only injury of the *axons* was by crushing, and it is therefore demonstrated that the larger number of small fibres is due to interruption of the whole nerve and its tubes.

In the posterior tibial nerves at the heel the fibres were distinctly larger on the side with the shorter nerve (Pl. 5, figs. 13, 14; 1010 *j, s*). On both sides, however, they were larger than after 107 days.

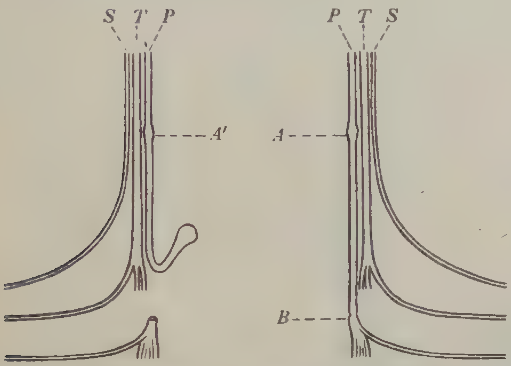
Sections of the crushed points showed similar conditions on the two sides (Pl. 5, figs. 15, 16; 1010 *a, k*). The unions between the peroneal and tibial nerves had been rather less successful than in rabbit 1011 (Pl. 6, figs. 17, 18; 1010 *f, n*). In particular, that on the side with the lengthened nerve showed a considerable lateral deviation of fibres. Though the stumps were closely apposed, conditions had evidently not been favourable for the formation of a clot with good longitudinal orientation. The most probable explanation for this is that the union was made *without longitudinal tension*, and the case is indeed a warning of the bad results which may follow from a union made under apparently ideal surgical conditions (see Weiss, 1943). The difference between the two suture lines may explain the somewhat smaller fibres found immediately below the crush in the shorter nerve.

Conclusions from the results of regeneration through artificially lengthened nerves. We may conclude from these two experiments that the length of nerve to be regenerated below an injured point does not greatly affect the rate of increase of diameter of the fibres in the section of nerve immediately below the injury. Maturation does not proceed uniformly along the whole length of a regenerating fibre, but spreads centrifugally, so that at any given time more distant points show a smaller diameter (see also Gutmann & Sanders, 1943).

Effect of absence of peripheral connexion on fibre diameter

Two rabbits (1012 and 1013) were operated on in such a way as to reveal the effect of presence or absence of a connexion with the periphery on regeneration in the sensory and motor bundles of the peroneal nerves. On each side the nerve was crushed in the upper third of the thigh, above the point at which it divides into separate bundles destined to supply muscles and skin respectively. The former bundles of course contain somatic motor as well as sensory fibres, whereas the cutaneous branches (anterior tibial nerve) contain sensory and sympathetic fibres only, the latter being almost entirely non-medullated. The nerve was also severed below the knee, where it passes under the lateral head of the gastrocnemius. On one side the cut lower ends were immediately reunited with plasma, whereas on the other side the severed end of the peroneal was turned aside and fixed in such a manner as to allow no possibility of reunion.

The animals were left to regenerate for 172 and 200 days and counts then made of the number of fibres in the sensory and motor bundles below the crush and above the point of severance. Table 2 shows that in both animals the number of fibres was greater and their diameter smaller in the nerves which were not connected with the periphery. In both cases these differences were found to be statistically significant. The table also shows the numbers and diameters of the fibres in the central stumps, above the region of crushing. In rabbit 1012, which had been allowed to regenerate for 172 days, there were 4986 fibres in the central stump on the side with a peripheral union, and 5097 on that with none, but the fibres were distinctly smaller in the latter. This is in itself a very interesting fact, suggesting that the recovery in diameter of central fibres following their decrease during regeneration is influenced in some way by functional connexion with the periphery.



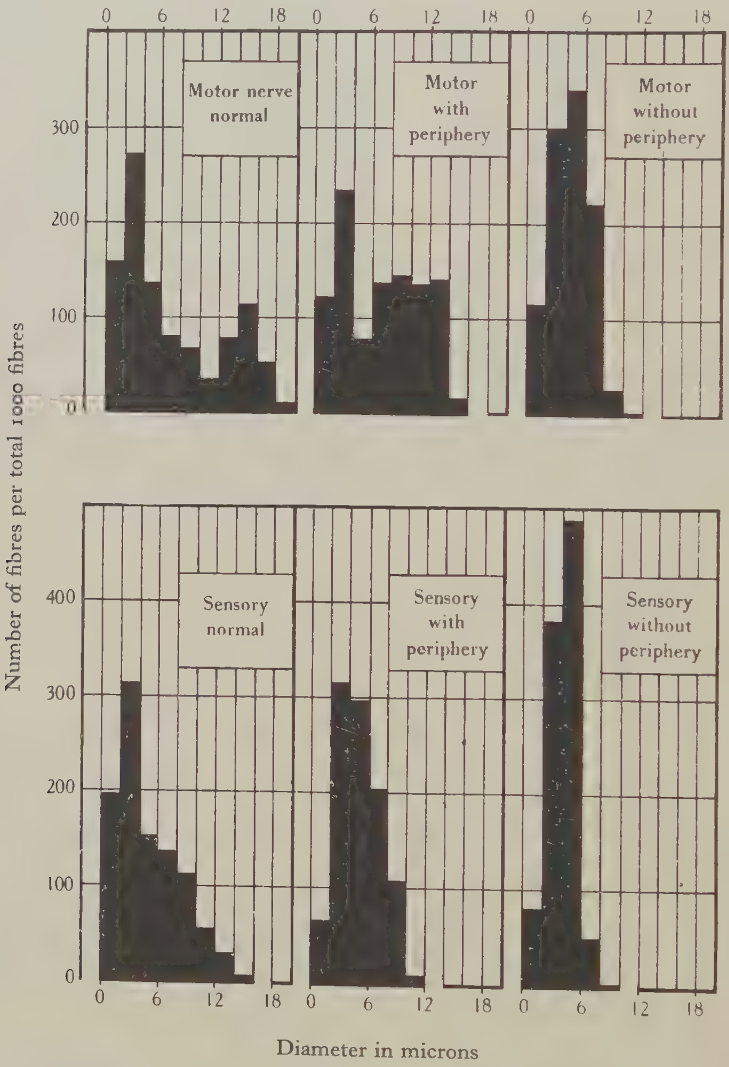
Text-fig. 2. Diagram of experiment to show effect of presence and absence of peripheral connexion on regeneration. The peroneal nerve is crushed in the thigh at *A* or *A′*. It is cut at the knee and on one side the central end is turned forward so that no union is possible. On the other side the stumps are joined at the lower level *B*.

Below the crushed point in the nerve connected with the periphery, there were 5361 fibres in motor and sensory bundles together, that is to say, only a few more than in the central stump. The diameter was distinctly larger in the motor than in the sensory branches (Pl. 6, figs. 19, 20). The regenerated stretch of nerve which had not been allowed to make connexion with the periphery contained a total of 11,213 medullated fibres, more than twice as many as in the central stump. This confirms the finding of Simpson & Young (1945) that when regenerating fibres fail to make contact with the periphery, far more of them are found in the peripheral than in the central stump. Weiss *et al.* (1945) have recently shown a similar effect in the nerves of rats. Further, the diameter of the regenerated fibres remains very small in this nerve, and *D is not greater in the motor than in the sensory bundles* (Pl. 6, figs. 21, 22). This last is a result of the utmost importance, since it strongly suggests that the factor which makes somatic motor fibres large is a peripheral, or 'functional' one. For since the upper lesion is a simple crush of the nerve, it is to be presumed that the large central fibres proceeded down large peripheral tubes and yet failed to reach a large size. Examination of the sections indeed shows the large tubes of the motor branches most clearly, each filled with numerous small fibres (Pl. 6, figs. 21, 22; 1012 *c*, sensory and motor). The sensory and motor branches can still be distinguished by the large tubes of the latter, in spite of the fact that the fibre diameters are similar in both. Histograms reinforce the pictures, showing that not only is the fibre diameter similar in the two nerves, but further that the large motor fibres have not given rise to any large fibres between the point of crushing and the neuroma, the maximum diameter being 12 μ in the motor and 10 μ in the sensory bundles. However, the motor bundles contain a larger number of fibres 6–10 μ in diameter than the sensory bundles, giving some indication of the operation of factors other than peripheral connexion.

Table 2. *The effect of peripheral connexion upon fibre maturation*

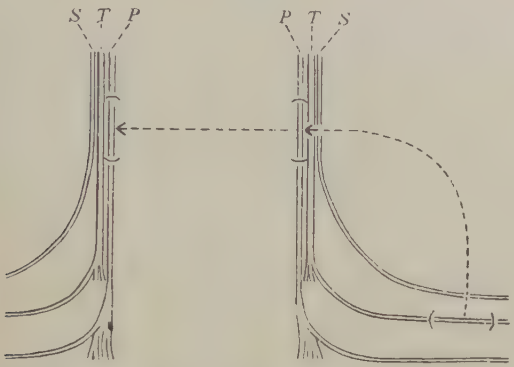
Animal	Days	Type of nerve	Periphery	Level	No. of fibres	*D* in μ	Total vol. of fibres cu.mm.
1012 *e*	172	Mixed	Connected	Central	4986	8·4	27·64
1012 *a*	172	Mixed	Unconnected	Central	5097	7·4	21·92
1012 *g*	172	Motor	Connected	Peripheral	2898	7·2	7·67
1012 *c*	172	Motor	Unconnected	Peripheral	7757	4·6	5·80
1012 *g*	172	Sensory	Connected	Peripheral	2463	5·2	10·20
1012 *c*	172	Sensory	Unconnected	Peripheral	3456	4·8	2·81
1013 *a*	200	Mixed	Connected	Central	5475	8·5	31·07
1013 *f*	200	Mixed	Unconnected	Central	5148	6·9	19·25
1013 *c*	200	Motor	Connected	Peripheral	1883	8·2	6·46
1013 *i*	200	Motor	Unconnected	Peripheral	6257	4·9	5·31
1013 *c*	200	Sensory	Connected	Peripheral	2885	5·5	13·37
1013 *i*	200	Sensory	Unconnected	Peripheral	2588	4·3	1·69

The second animal, 1013, shows essentially similar results. Here the total number of fibres below the crush on the side in which connexion with the periphery was allowed was 4768, slightly less than in the central stump (5475). In the nerve with no peripheral connexion the number of regenerated fibres was again much greater (8845) than that in the central stump (5148). The central stump fibres were, as before, smaller in the nerve with no periphery. Their reduction in diameter without, apparently, reduction in thickness of their myelin sheaths, leads to the production of the remarkable fibres seen in Pl. 7, fig. 24 (1013 *f*) [cf. fig. 23 (1013 *a*)]. The fibres of the motor bundles which had been allowed to make functional connexions showed a typical increase in diameter, and there had been some increase in diameter also in the sensory bundles which had made a peripheral connexion. In the motor bundles with no peripheral connexion *D* was at most only slightly greater ($4\cdot9\,\mu$) than in the sensory bundles ($4\cdot3\,\mu$). The results from this experiment, therefore, fully confirm that of the previous one in showing that all regenerated fibres remain small unless connected with the periphery, such connexion causing some increase of diameter in sensory and still more in motor fibres.



Text-fig. 3. Histograms to show the number of fibres of different sizes in normal motor and sensory branches of the peroneal nerve, and in these same branches after 200 days of regeneration below a crushed point, with and without connexion with the periphery.

Regeneration of fibre diameter in grafts of nerves containing fibres of different diameters. In the experiments of Simpson & Young (1945) it was shown that pieces of splanchnic nerve grafted to post-intercostal nerves came to contain quite large fibres. The present series included two animals in which a test of the same sort was made by grafting into the peroneal nerve in the thigh. On one side of each animal, the graft was a piece of the peroneal from the opposite side. The defect made by taking this graft was then filled by a piece of posterior tibial nerve from the same animal.



Text-fig. 4. Grafting experiment to show the effect of sizes of tubes on the diameter reached by regenerating nerve fibres. On one side a portion of the posterior tibial nerve (small fibres) is grafted to the peroneal nerve in the thigh, the piece removed from this nerve (large fibres) is then grafted to the peroneal on the opposite side.

The posterior tibial contains smaller medullated fibres than the peroneal (see Sanders & Young, 1944), and the experiment therefore tests the extent to which the large fibres present in the peroneal nerve in the thigh are able to dilate the small tubes of the posterior tibial when they have passed through the latter and made connexion with the peroneal muscles. The grafts were 2 cm. in length and were fixed in place with clotted cockerel plasma.

The first animal (1015) was allowed to survive for 98 days, but the experiment was reduced in value by the fact that the upper union of peroneal with posterior tibial was poorly made, the two stumps being separated by about 0·5 mm. of irregular connective tissue. Very many nerve fibres had nevertheless penetrated this, and the grafted bundles of the posterior tibial contained many medullated fibres. Those in the corresponding piece of grafted peroneal nerve on the opposite side were more numerous, but perhaps not larger. However, in view of the uncertainties introduced by the poor union no counts were made. Below the grafts both peroneal nerves contained many medullated fibres, that with the peroneal nerve graft containing more than that with the posterior tibial graft. This case, therefore, shows that in spite of a poor union the many small-fibred bundles of the posterior tibial, when used as a graft, can allow a connexion to be made.

The second animal (1037) was allowed to survive for 200 days after an exactly similar operation. Here the union of the peroneal and posterior tibial graft had been well made (Pl. 7, fig. 25; 1037 *b*), though the presence of many bundles had led to some complications. However, it may reasonably be assumed that the opportunities for connexion were similar to those presented by the union of the peroneal with the peroneal graft (Pl. 7, fig. 26; 1037 *h*) on the opposite side. The lower unions of both grafts with the peroneal nerves were also well made.

Table 3 shows that the fibres were somewhat larger in the graft of the peroneal than in that of the posterior tibial (Pl. 7, figs. 27, 28). In the peroneal the difficulties of non-uniformity of various parts of the graft, owing to poor fixation in the centre, make the difference in fibre diameters observed of doubtful significance. Certainly the small tubes of the posterior tibial graft have not grossly restricted the growth of the fibres, but the result suggests that they have done so to some extent.

Table 3. *The effect of tube size on fibre maturation*

Animal	Days	Source of graft	No. of fibres	D in μ	Probability
1037 *i*	200	Peroneal	7300	5·9	0·1
1037 *c*	200	Posterior tibial	9377	5·5	

No strict comparison is possible between the diameters here recorded and those shown in Tables 1 and 2, which are of fibres regenerating below a point of crushing and not one of suture as in the present case. However, it is interesting that the diameter reached by the fibres is distinctly above that found in those nerves which made no connexion with the periphery. Evidently, after passing through the grafts, the fibres have been able to make connexion with their end-organs and to undergo a subsequent increase in diameter. Below the grafts fibres of large diameter were found on both sides (Pl. 7, figs. 29, 30).

DISCUSSION

These quantitative results abundantly confirm the previous evidence that fibres become large if they make connexion with the periphery (Weiss & Taylor, 1944; Sanders & Young, 1945; Weiss *et al.* 1945). Regenerating stretches of nerves which end blindly contain far more and smaller fibres than others comparable in every way except that the fibres can make connexion with muscles and skin. A very important new result which emerges from these counts is that regenerating motor fibres which do not reach their end-organs become very little larger than sensory fibres. This shows the power of the peripheral effect very strikingly, and suggests that it is of paramount importance. It seems not unlikely that during normal development all fibres are of like diameter, and that the spectrum of fibre sizes develops as a result of the peripheral connexions, perhaps by the very act of functioning. Indeed, it may well be that the influence extends back from the fibres to the cell-bodies themselves, and that it is the peripheral connexion which determines the size of cells in the spinal cord (see Young, 1945). Such an effect of the periphery upon the centres recalls the theories of Weiss. He has always maintained that such an influence exists, though not quite in the sense now revealed.

The figures of Table 2 show for the first time that sensory as well as motor fibres are affected by their connexions with the periphery. The anterior tibial nerve of the rabbit is a pure skin nerve (see Sanders & Young, 1944), its only motor fibres being sympathetic, presumably non-medullated and therefore not considered here. Yet during regeneration its fibres become larger if they are allowed to reach the periphery. It should perhaps be noted, however, that since the suture at *S* of Text-fig. 2 is of the *whole* peroneal nerve, many of these sensory fibres will enter the peroneal muscles. However, Gutmann (1945) has confirmed the previous evidence that sensory fibres cannot form motor end plates in muscle. Further evidence about the factors which control the maturation of sensory fibres is needed: these experiments show that the peripheral connexions exercise a large influence.

The present investigations still leave it quite uncertain how connexion with the periphery comes to exert its effect on maturation of the fibres. The experiments in which the nerves were artificially increased in length show that the diameter reached by fibres immediately below a crushed point is not greatly affected by the length of nerve to be regenerated more distally.

Hand in hand with the increase in size of the fibres which have become connected with the periphery goes the reduction and disappearance of those remaining without connexion, but it is impossible at present to say whether the one process controls the other. The growth of one fibre in a tube may have the effect of constricting the others and hence leading to their degeneration. Until such degeneration has been actually observed it is impossible to say more. It may be that the material used to make the large fibres is in some way derived from the others arising from the same parent fibre, thus producing their degeneration. Certainly the whole increase produced by peripheral connexion is not obtained by draining material from other fibres. For the total volume occupied by the fibres in the peripheral stump of an unconnected nerve is much less than that of the fewer, but larger, fibres which develop when a peripheral connexion is allowed. Weiss *et al.* (1945) also found this in the nerves of rats. The last column of Table 2 contains estimates of the volumes occupied by fibres in the central and peripheral stumps of the nerves. Assuming that the distance from the point of crushing to the neuroma was 45 mm., to the peroneal muscles 65 mm., and to the skin 195 mm., the total volume of the fibres in the peripheral stump is seen to be very much greater in the nerves connected with the periphery. The total increase of some of the fibres cannot therefore have been only at the expense of others. Moreover, it must be remembered that the fibres *central* to the crush also remain small in an unconnected nerve. Assuming that the diameters found immediately above the lesion are maintained to the cord, the volume in the central stumps would be 28 and 31 cu.mm. in the nerves connected with the end-organ, but only 22 and 19 cu.mm. in those ending blindly as neuromas, assuming that all crushes are made at a distance of 100 mm. from the cord. Weiss *et al.* (1945) have already noted that the fibres remain small in the central stumps of a regenerating nerve without peripheral connexion. As they observe, the small size of such fibres cannot be due entirely to drainage of axoplasm from the central fibres. The volume of the many small fibres in an unconnected regenerating nerve is smaller than in a similar nerve having connexion with the periphery (see Table 2), whereas the central stump of the latter contains the larger fibres. Hammond & Hinsey (1945) believed that the small decrease which they observed in the central stumps of sutured nerves might be due to the presence of many small fibres turned back at the junction. A similar explanation cannot apply in the present case, since the number of fibres in the central stump of the unconnected nerves was not significantly greater than in those with a peripheral connexion. However, it seems likely that some part of the large number of fibres seen above the neuromas was due to 'doubling back' towards the centre as suggested by Weiss *et al.* (1945).

It is impossible to resist the conclusion that the making of a connexion with the periphery produces an actual increase in the size of the fibres, whereas without such connexion not only do newly regenerated fibres not increase, but also their parent fibres remain small. Functional hypertrophy is, of course, common enough in muscles, glands, and other tissues, and it is most interesting to find it also in a tissue whose function is conduction. It is much to be hoped that it will be possible to throw further light on the processes by which an effect of such importance for the functioning of the organism is produced.

SUMMARY

1. Stretches of regenerating rabbit's nerve in which no connexion with the periphery is allowed contain numerous and small nerve fibres.

2. Both cutaneous sensory and motor nerve fibres become large when allowed to reach their end-organs, but the motor fibres attain greater diameters than the cutaneous sensory fibres.

3. Motor fibres regenerating without opportunity of reaching muscle fibres become little if any larger than sensory fibres when both are unable to reach their end-organs. It is, therefore, probable that the peripheral connexion is of great importance in determining fibre diameter.

4. The fibres of the central stump of a nerve regenerating unconnected with the periphery remain small and have relatively very thick myelin sheaths.

5. The diameter of nerve fibres regenerated in the region below a crushed point on a nerve is not influenced by the length of nerve to be regenerated distally.

6. Increase of diameter and medullation proceeds centrifugally from a lesion, more distant points showing a lesser degree of maturation at any given time.

7. Fibres regenerated in a grafted piece of posterior tibial nerve were only slightly smaller than those in a grafted piece of peroneal nerve. Connexion with the periphery is, therefore, able to produce an increase in fibre diameter which overrides any constriction of fibre growth produced by the slightly smaller Schwann tubes.

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DESCRIPTION OF PLATES

The photographs of Weigert preparations of transverse sections are direct bromide prints at a magnification of 430 diameters.

The photographs of the junctions are of preparations stained by Bodian's protargol method at a magnification of 21 diameters.

Plate 4

Fig. 1. Fibres below crushed point above lengthened peroneal nerve. 107 days (1011 *i*).
Fig. 2. Fibres below crushed point on the opposite side where the nerve is not lengthened (1011 *d*).
Fig. 3. Lower down in lengthened peroneal nerve (1011 *k*).
Fig. 4. Lower down in peroneal nerve, not lengthened (1011 *f*).
Fig. 5. Posterior tibial nerve supplied with fibres from peroneal which have run a long course (1011 *l*).
Fig. 6. Posterior tibial nerve on opposite side supplied with peroneal fibres without lengthening (1011 *m*).
Fig. 7. Junction of peroneal with tibial nerve on lengthened side. Peroneal to left (1011 *j*).
Fig. 8. Junction of peroneal with tibial nerve without lengthening. Peroneal to left (1011 *e*).

Plate 5

Fig. 9. Fibres immediately below crushed point of lengthened peroneal nerve. 200 days (1010 *c*).
Fig. 10. Fibres of opposite crushed peroneal nerves, not lengthened (1010 *m*).
Fig. 11. Lower down in lengthened peroneal nerve (1010 *g*).
Fig. 12. Fibres lower down in peroneal nerve, not lengthened (1010 *q*).
Fig. 13. Posterior tibial nerve supplied with fibres for 200 days from lengthened peroneal nerve (1010 *j*).
Fig. 14. Fibres of posterior tibial nerve supplied from peroneal nerve without lengthening (1010 *s*).
Fig. 15. Crushed point of lengthened peroneal nerve (1010 *a*).
Fig. 16. Crushed point of peroneal nerve not lengthened (1010 *k*).

PLATE 6

Fig. 17. Union of peroneal with tibial nerve to give increased length (1010 *f*). Peroneal to right.

Fig. 18. Union of peroneal with tibial nerve without increase of length (1010 *n*). Peroneal to right.

Fig. 19. Fibres in motor branch of peroneal nerve below crush which have been allowed to reach periphery. 172 days (1012 *g*).

Fig. 20. Fibres of sensory branch of peroneal below crush which have been allowed to reach the periphery (1012 *g*).

Fig. 21. Fibres of motor branch of peroneal nerve below crush which have not been allowed to reach periphery (1012 *c*).

Fig. 22. Fibres of sensory branch of peroneal nerve below crush which have not been allowed to reach periphery (1012 *c*).

PLATE 7

Fig. 23. Fibres of central stump of peroneal nerve which have been crushed and allowed to reach periphery. 200 days (1013 *a*).

Fig. 24. Central stump on opposite side above crush without union with periphery (1013 *f*).

Fig. 25. Point of union of peroneal nerves with grafted posterior tibial nerve. 200 days (1037 *b*). The graft lies to the right.

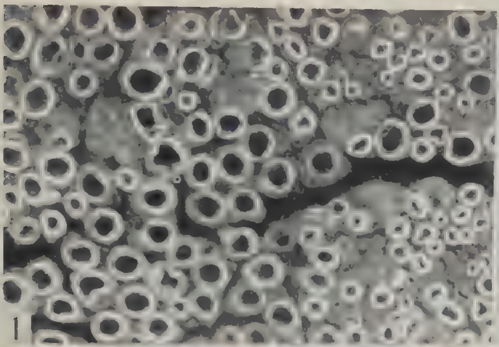
Fig. 26. Point of union of peroneal nerve with grafted piece of peroneal (1037 *h*). The graft is to the right.

Fig. 27. Fibres in piece of posterior tibial grafted into the peroneal (1037 *c*).

Fig. 28. Fibres in peroneal graft to peroneal (1037 *i*).

Fig. 29. Fibres in peroneal nerve below posterior tibial graft (1037 *e*).

Fig. 30. Fibres in peroneal nerve below graft of peroneal (1037 *k*).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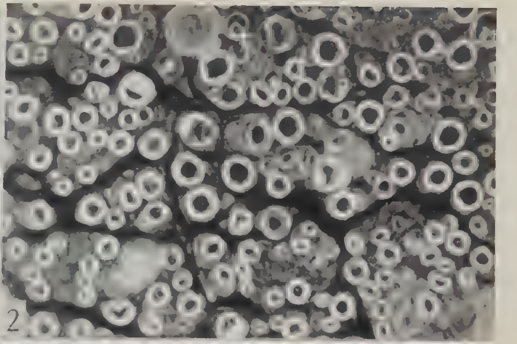
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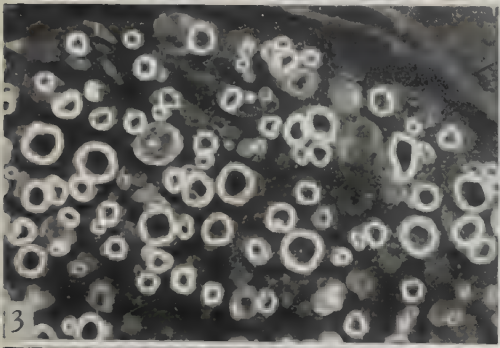
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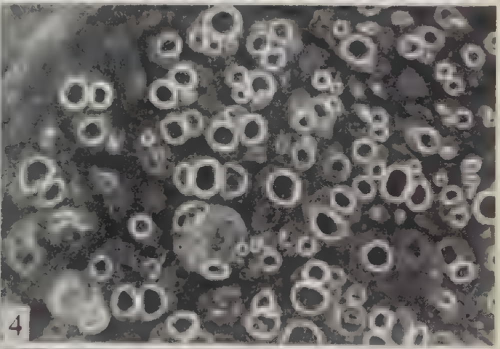
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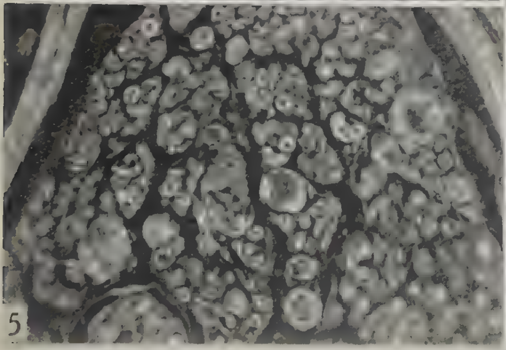
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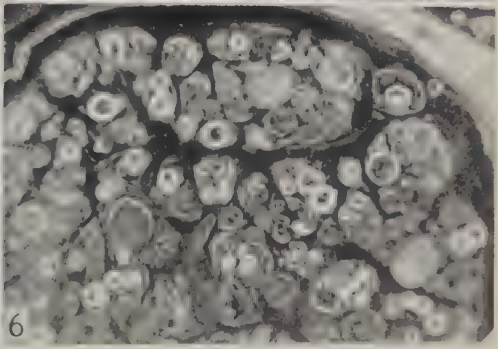
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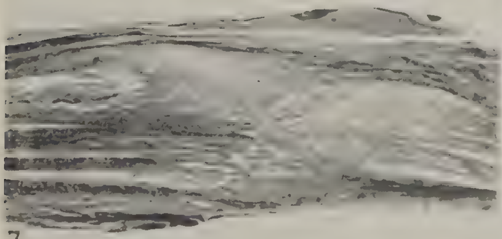
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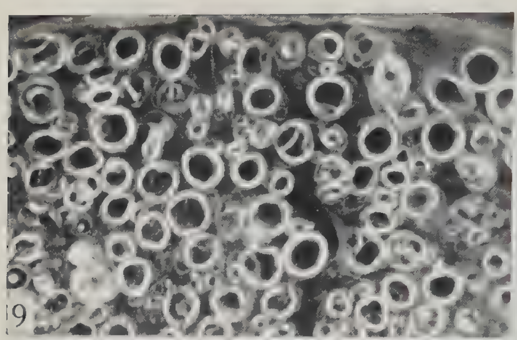
SANDERS AND YOUNG—THE INFLUENCE OF PERIPHERAL CONNEXION ON THE DIAMETER OF REGENERATING NERVE FIBRES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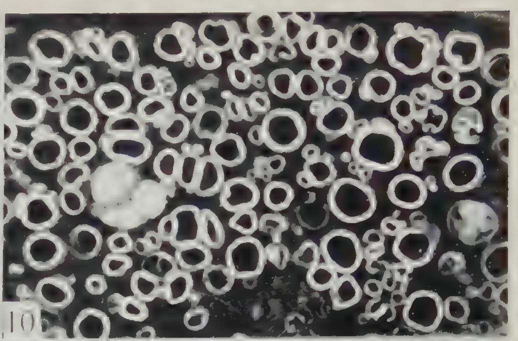
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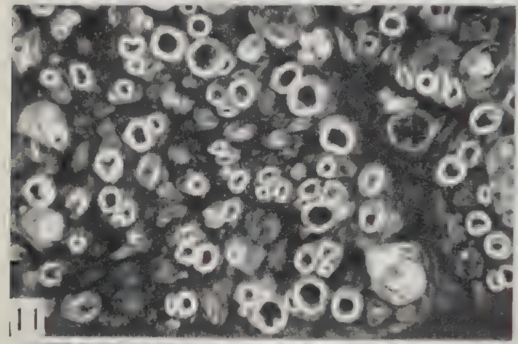
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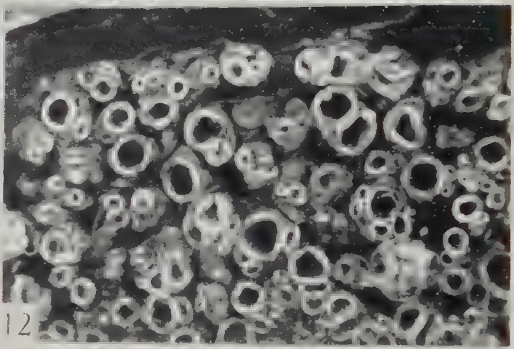
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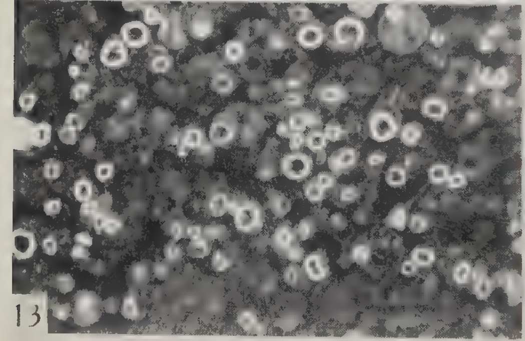
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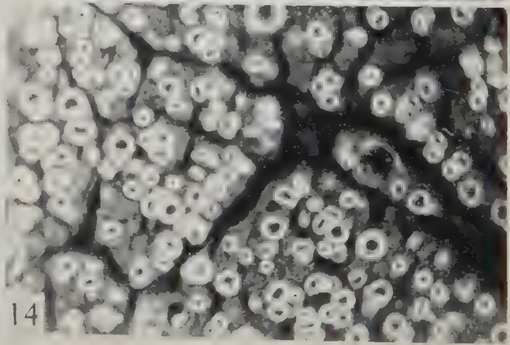
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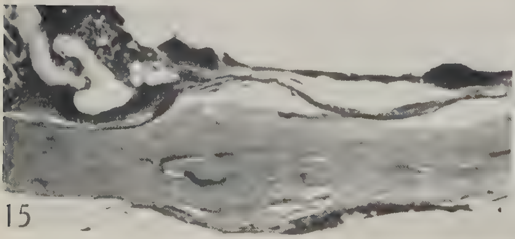
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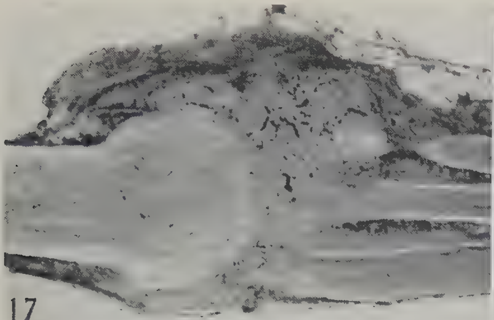
SANDERS AND YOUNG—THE INFLUENCE OF PERIPHERAL CONNEXION ON THE DIAMETER OF REGENERATING NERVE FIBRES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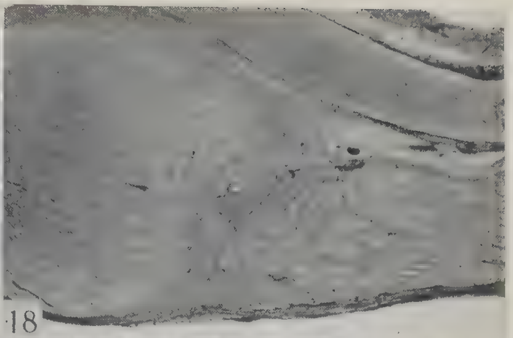
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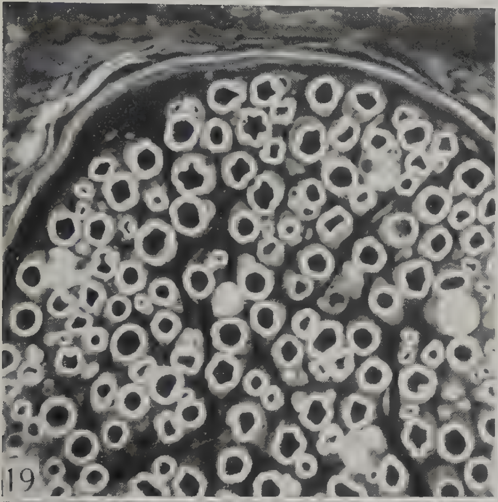
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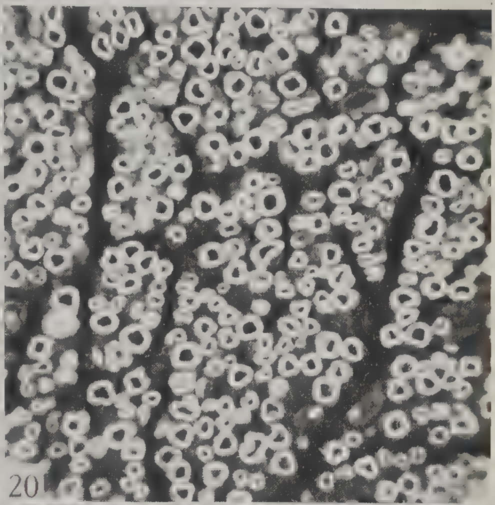
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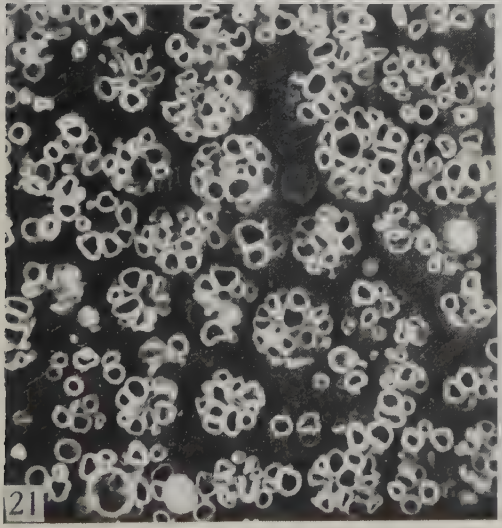
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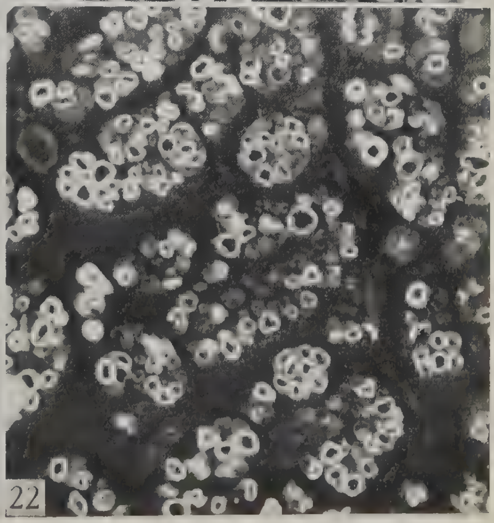
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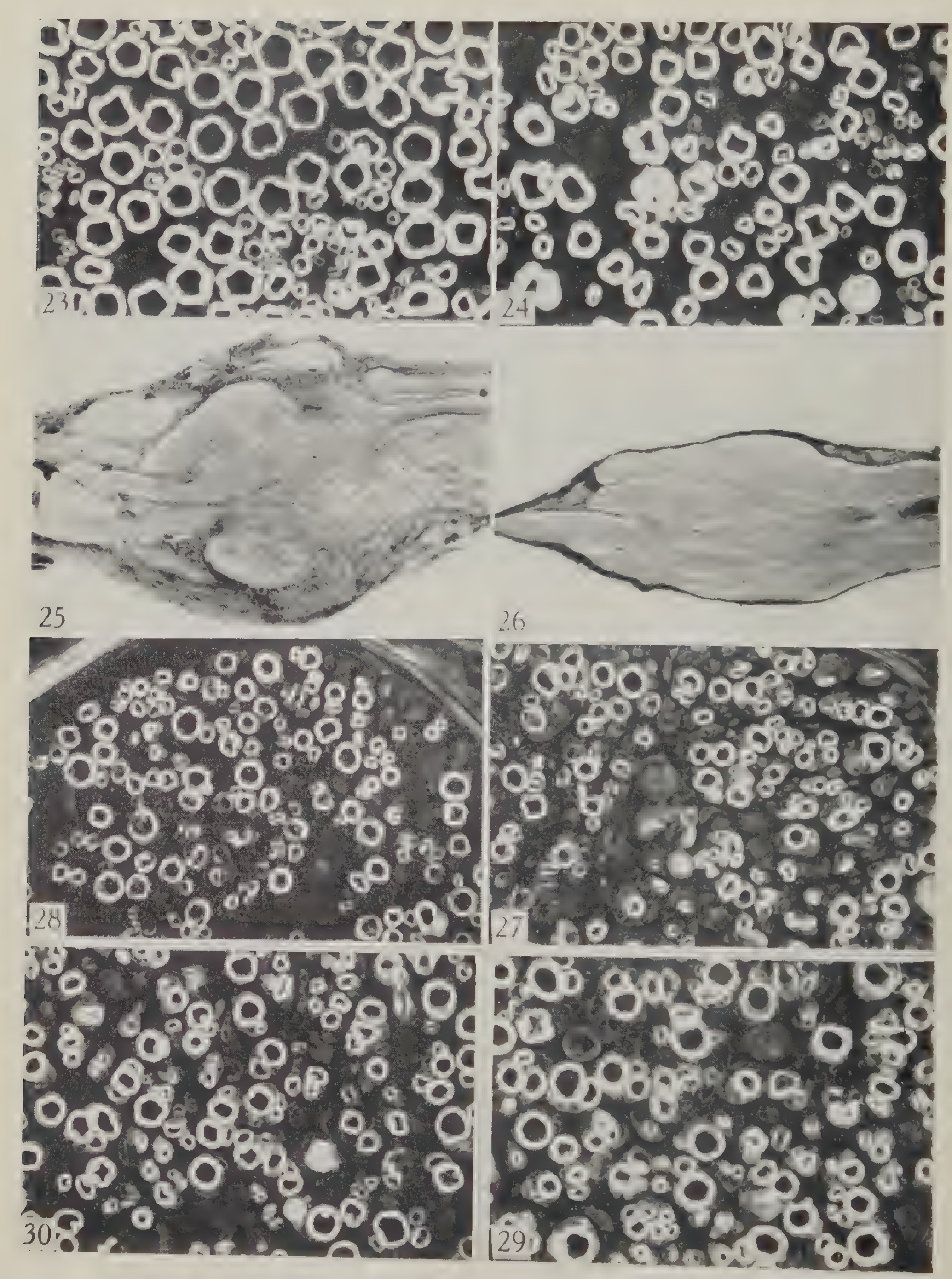
SANDERS AND YOUNG—THE INFLUENCE OF PERIPHERAL CONNEXION ON THE DIAMETER OF REGENERATING NERVE FIBRES



SANDERS AND YOUNG—THE INFLUENCE OF PERIPHERAL CONNEXION ON THE DIAMETER OF REGENERATING NERVE FIBRES

STUDIES ON TEMPERATURE REGULATION

II. AMPHIBIA AND REPTILES

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(*Received* 8 *January* 1946)

(With Four Text-figures)

1. INTRODUCTION

A previous communication (Hogben & Kirk, 1944*b*) drew attention to the fact that emergence of animal life on land offers a choice between two ways of solving a dilemma which arises as soon as animals expose themselves to climatic conditions of terrestrial habitats under adverse conditions of high air temperature and intense solar radiation:

(*a*) maintenance of body temperature below the lethal limit by evaporation of water from the surface;

(*b*) selection of stocks with a relatively high thermal death-point, hence able to penetrate dry land without restrictions imposed by such continuous obligatory loss of water.

Several authors (Gray, 1928; Hall & Root, 1930) have suggested that different rates of water loss, such as occur respectively among Amphibia and reptiles, furnish an important clue to this aspect of the evolution of terrestrial vertebrates. From their own observations Hall & Root conclude that the influence of external humidity changes on the body temperature of vertebrates (salamander, frog, toad, lizard, *Phrynosoma*, turtle and alligator) decreases as we pass from representatives of older to representatives of more recent groups; but none of these authors record how far changes of thermal death-point have accompanied increased resistance to desiccation. On some matters our own observations do not tally with those of Hall & Root on the body temperature of the frog (Hogben & Kirk, 1944*a*), and if the discrepancies are attributable to the apparatus they employed, similar observations carried out on other species merit reinvestigation. We have, therefore, felt it necessary to extend our investigations of the thermal death-point and body temperature of the frog (Hogben & Kirk, 1944*a*) to the toad (*Bufo bufo*), which is less restricted to a moist habitat, and to two representatives of reptilian stock, the alligator (*A. mississippiensis*) and the slow worm (*Anguis fragilis*). Since little is known about the effect of acclimatization on the thermal death-point of vertebrates it was felt advisable to confine our studies on thermal death to species indigenous to this country, and preferably to carry out experiments at the same season of the year. The number of reptile species easily obtainable is thus strictly limited. Of the two stocks we have kept (*Lacerta vivipara* and *Anguis fragilis*) the former suffered such a high natural mortality under laboratory conditions as to make assessment of its thermal death-point without recourse to a very large number of animals open to doubt. The slow worm, however, is easy to keep in the laboratory, and is extremely viable even when starved for long periods. Out of a stock of 54 animals only 1 died during the course of 2 months.

2. METHODS

Two types of experimental chamber were constructed for the temperature investigations recorded below. The first, essentially the same as one previously described (Hogben & Kirk, 1944*b*), was used for the experiments on the toads and slow worms. A much larger chamber was constructed for the alligators. A square galvanized iron tank (37 × 80 × 89 cm.) was fitted with a lid, half wood and half glass to facilitate inspection of the animal during the course of an experiment. The tank was heated with a 1 kW. electric fire element, so arranged with baffles that no direct radiation fell on the animal; cooling pipes from a refrigerating unit were fitted into one corner. Humidity was controlled by means of trays of $CaCl_2$, water and $H_2O : H_2SO_4$ mixtures in the bottom of the tank, and the air was kept in rapid motion by means of a fan attached to the lid. To prevent excessive movement during an experiment the alligator was placed in a wide-mesh wire cage held on two supports inside the chamber so that air could circulate freely round the whole body of the animal. Conduction from the substratum was thus reduced to a minimum. Air temperatures, wet-bulb temperatures and body temperatures were measured with needle thermocouples manufactured by the Cambridge Instrument Company. For the purpose of measuring body temperature the thermocouple was driven into the musculature between the scutes about 2 in. posterior to the cloaca. Damage to the visceral organs was thereby avoided whilst permitting a fair assessment of the temperature of the main body region.

Special rectal thermocouples were constructed for the measurement of the body temperature of the slow worms. A copper-constantan junction (25 and 28½ s.w.g. respectively) was cemented with soft solder into one end of a silver tube of external diameter 0·3 cm. and length 4 cm. The leads ran back through the tube and were encased in thin rubber drainage tubing which was slipped over the other end of the silver tube. A cold junction was made closely identical to the two rectal thermocouples. They were used in conjunction with a high sensitivity short period galvanometer which was critically damped. The circuit was far more stable than with any of the types of thermocouple previously employed and readings accurate to 0·1° C. could be repeated after long intervals. During an experiment one of the thermocouples was introduced into the rectum of the slow worm and kept in position by binding the lead to the tail with cotton immediately behind the cloaca. The animal was then allowed to move freely around the experimental chamber moving over the surface of a perforated zinc plate raised 2 in. from the bottom of the chamber. The air temperature at the surface of the plate was measured with the other silver thermocouple and the wet-bulb temperature of the circulating air just above the surface of the plate determined by a mercury in glass thermometer graduated with divisions of 0·1° C., the bulb of which was surrounded with a wad of wet cotton-wool. Humidity control was effected by means of trays of $H_2O : H_2SO_4$ mixtures in the bottom of the experimental chamber and in the large air reservoir from which air was drawn by the circulating fan.

3. TEMPERATURE REGULATION OF AMPHIBIA

(*a*) *Thermal death-point.* The mortality of toads (*Bufo bufo*), obtained from dealers, was investigated by allowing them to swim in water for a period of 1 hr., the temperature of the water being accurately controlled to ±0·1° C. After immersion the toads were placed in clean moist dishes kept at room temperature and the mortality at the end of 24 hr. was noted. The body temperature during the period of immersion was taken to be the same as that of the water. In all, 95 toads were used and the results of these experiments are recorded in Table 1. It would appear from an examination of this table that there is little difference between the thermal death-point of *B. bufo* and that which we had previously determined for *Rana temporaria*. A further series of observations on *R. temporaria* was carried out at the higher temperatures. Combined with previous observations this provides the material for Table 2.

Table 1. *April*

Temperature operative period 1 hr. Examined 24 hr. later

Temp. of water ° C.	No. alive after 24 hr.	No. dead after 24 hr.
27·7	4	0
30·0	4	0
31·5	7	0
32·0	6	2
32·5	14	6
33·0	7	1
33·5	5	16
34·0	0	8
34·5	1	10
35·5	0	4
Total	48	47

Table 2. *April*

Temperature operative period 1 hr. Examined 24 hr. later

Temp. of water ° C.	No. alive after 24 hr.	No. dead after 24 hr.
30·1	10	0
31·0	10	0
31·5	7	0
32·0	8	2
32·5	12	6
33·0	2	8
33·5	0	18
Total	49	34

Table 3. *Bufo bufo*

Air temp. ° C.	R.H. %	Body temp. T_1° C.	Wet-bulb temp. T_2° C.	$T_1 - T_2$° C.
30·9	24	20·7	17·1	+3·6
30·8	38	22·6	20·5	+2·1
29·8	50	24·4	22·0	+2·4
29·2	49	23·1	21·5	+1·6
27·5	27	17·5	16·0	+1·5
27·5	41	20·1	18·7	+1·4
27·6	82	26·5	24·9	+1·6
26·8	58	22·5	20·8	+1·7
24·1	26	15·1	13·2	+1·9
24·8	34	16·9	15·3	+1·6
23·7	26	14·5	13·2	+1·3
19·5	49	14·6	13·4	+1·2
21·4	86	19·8	19·7	+0·1

Under the experimental conditions described above it has not been possible to keep frogs of the species *R. temporaria* alive after immersion for 1 hr. in water at 33·5° C. An appreciable number of toads do survive, however, at this temperature. Fig. 1 exhibits rough mortality graphs for *B. bufo* and *R. temporaria* based on the data in Tables 1 and 2. They suggest a slightly greater resistance of *B. bufo* to thermal death, but the difference for 50 % mortality is only of the order of 0·5° C.

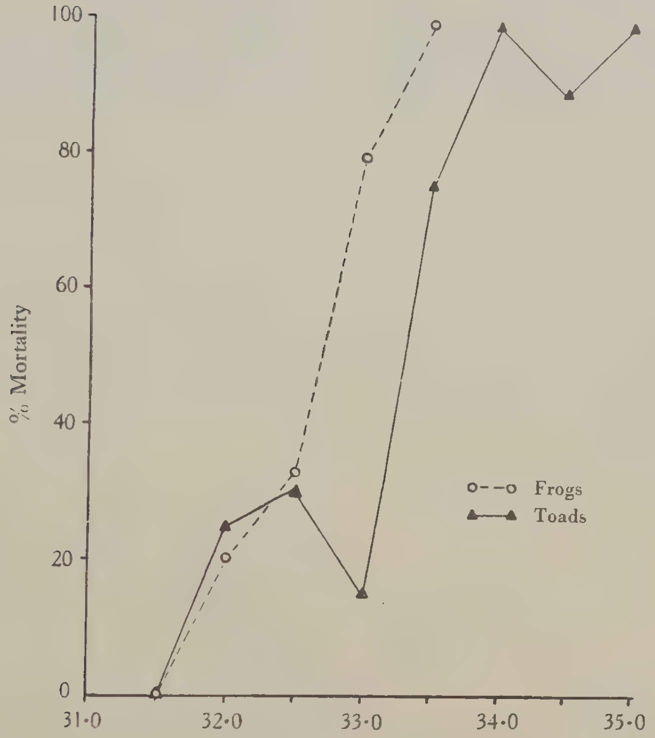
100
80
60
40
20
0
% Mortality
31·0
32·0
33·0
34·0
35·0
Operative temperature ° C.
Frogs
Toads

Fig. 1.

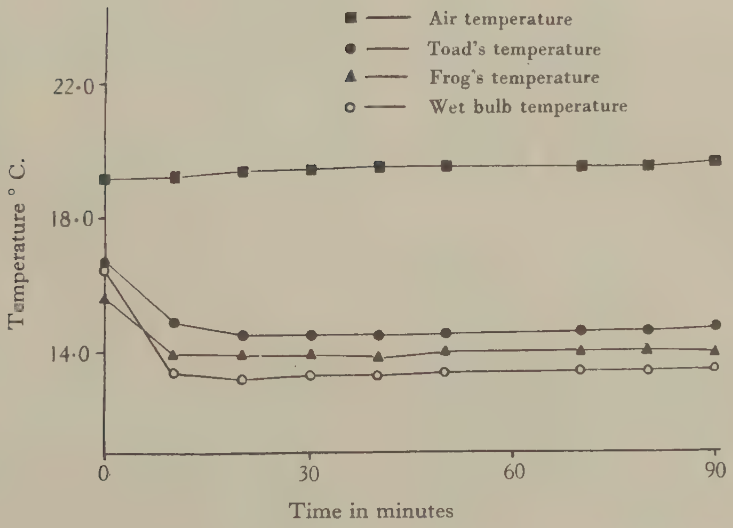
Air temperature
Toad's temperature
Frog's temperature
Wet bulb temperature
22·0
18·0
14·0
Temperature ° C.
0
30
60
90
Time in minutes

Fig. 2.

(*b*) *Body temperature.* Table 3 gives the results of a number of experiments at different air temperatures and humidities. At low humidities the body temperature of the toad, like that of the frog, is considerably below that of its surroundings, though generally well above that of the wet-bulb thermometer. In other experiments, recorded in Table 4, the body temperature of toads and frogs was measured at the same time, the animals being placed side by side in the experimental chamber. Care was taken to select animals of the same sex and of approximately the same weight. A direct comparison of the temperature response of frogs and toads to different environmental conditions is thus possible, and a typical experiment is shown in Fig. 2.

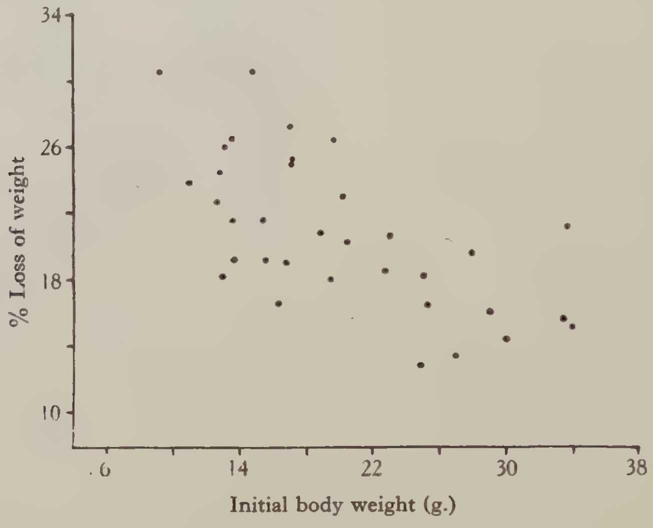

Fig. 3.

Table 4. *Differences between rectal temperature of frogs and toads*

$\Delta t = t_{\text{toad}} - t_{\text{frog}}$

Time (min.)	1	2	3	4	5	6	7
30	+0·2	0·0	−0·7	+0·9	−1·0	+0·6	+0·2
40	+0·3	0·0	−0·7	+0·8	−0·9	−0·1	+0·1
50	0·0	0·0	−0·5	+0·9	−0·8	−0·1	0·0
60	+0·3	0·0	—	+0·9	−0·6	−0·1	+0·1
70	+0·4	−0·3	−0·8	+0·7	−0·5	+0·7	0·0
80	+0·3	−0·3	−0·6	+0·8	−0·5	+0·8	−0·1
90	+0·2	−0·3	−0·6	+0·4	−0·4	−0·8	+0·6
Mean air temp. °C.	32·0±0·1	27·4±0·4	21·4±0·2	24·6±0·2	27·2±0·2	26·6±0·3	29·2±0·3
Mean rectal temp. toad °C.	20·4±0·4	26·4±0·4	19·8±0·2	16·7±0·3	17·2±0·3	22·3±0·3	23·0±0·2
Mean wet-bulb temp. °C.	17·0±0·1	24·8±0·2	19·7±0·2	15·3±0·0	15·8±0·2	20·9±0·1	21·6±0·1
R.H. %	36	92	86	43	36	72	59

In individual experiments the body temperature of the toad might be slightly in excess of that of the frog, or vice versa, but the mean value of Δt for all the experiments is zero. From the results of these experiments, therefore, it would seem that the toad loses water as freely as the frog, although there are individual variations in both species. This conclusion does not agree with the work of Hall & Root referred to above. In general their figures show that at 20° C. the depression of body temperature of the toad in relative humidities of 0–75 % is only four-fifths that of the depression of the body temperature of the frog at a corresponding R.H. It is possible that these discrepancies may be due to the different species which were used by the American investigators. The problem of the small intra species variations in body temperature was subject to further investigation in the case of the frog. A large number of animals was dehydrated under standard conditions, weights being recorded at regular intervals. Each frog was removed from water, and its bladder emptied by slight abdominal pressure. It was placed in a dry breffit jar covered with a light muslin top and kept at 25° C. and 75 % R.H. in turbulent air. The scatter diagram of Fig. 3 exhibits initial body weight plotted against percentage loss of weight during a 48 hr. period. As would be expected, it reveals a decrease with respect to rate of loss of water as body weight increases, but the trend is subject to considerable individual variability. If we assume that the thermal capacity of frogs' tissue remains roughly constant this variability with reference to rates of water loss should correspond to individual discrepancies with respect to body temperature within the same species in identical external circumstances.

4. BODY TEMPERATURE OF REPTILES

(*a*) *Upper thermal death-point.* The upper thermal death-point of slow worms (*Anguis fragilis*) was determined by placing them in closed moist Petri dishes, 23 cm. in diameter and 5 cm. deep, in a thermostatically controlled incubator. The temperature inside the dish was measured by a mercury in glass thermometer inserted through the lid. A double-walled glass door fitted to the incubator facilitated observation of the animals during the course of an experiment. The air inside the dish was considered to be almost completely saturated with water vapour. At the beginning of an experiment four animals were removed from the tank in which they were kept and transferred rapidly to the Petri dish which was already at the desired temperature. At the end of 1 hr. the animals were transferred from the incubator to another closed Petri dish and kept at room temperature. Numbers alive and dead 24 hr. later are recorded in Table 5. From this it is clear that the temperature for 50% mortality in the laboratory stock is near 37·5° C. when the operative period is 1 hr. It is interesting to note that the state of *reversible* heat rigor such as affects the majority of frogs between 30 and 33° C. does not occur in slow worms. Almost invariably animals that fail to give eyelid response to mechanical stimulation at the end of the temperature operative period, also fail to recover during the next 24 hr.

(*b*) *Body temperature of the slow worms.* The body temperature of the slow worms under varying conditions of air temperature and humidity was determined by adopting the procedure already outlined in paragraph 2. The results of a series of experiments are recorded in Table 6. Twenty minutes after being placed in the experimental chamber the body temperature, as measured in the rectum, closely approximates that of the air. If the humidity of the air is low the body temperature is slightly below that of the air. At higher humidities and air temperatures above 20° C., cooling by evaporation is apparently insufficient to carry away all the metabolic heat and the body temperature is raised above that of the air. Briefly, the results of these experiments indicate that over a wide range of air temperature and humidity the body temperature of the slow worm at no time differs from that of the air by more than 1° C. We have made no attempt to evaluate the effect of the temperature of the substratum on the body temperature of the slow worm. Under experimental conditions the temperature of the metal plate on which the animal crawled was identical with that of the air. In the field such an agreement between air and ground temperatures is exceptional, and it is likely that the ground temperature is an important circumstance contributory to the body temperature of limbless tetrapods such as the slow worm and *Ophidia*.

Table 5. *October*

Temperature operative period 1 hr. Examined 24 hr. later

Temp. of air ° C.	No. alive after 24 hr.	No. dead after 24 hr.
35·0	4	0
35·5	4	0
36·5	4	0
37·0	5	7
38·0	1	7
Total	18	14

Table 6. *Anguis fragilis*

$\Delta t = t_{\text{air}} - t_{\text{rectum}}$ °C.

Air temp.	R.H. %	Δt after			Mean value of Δt° C.
		20 min.	30 min.	40 min.	
30·7	19	+0·5	+0·3	+0·2	+0·33
29·7	38	0·0	+0·2	+0·7	+0·30
28·4	64	−0·1	−0·4	−0·3	−0·27
26·8	18	+0·3	+0·3	+0·1	+0·23
23·1	71	−0·6	−0·6	−0·7	−0·63
21·6	27	+0·2	0·0	0·0	+0·07
17·3	45	+0·2	+0·2	+0·2	+0·20
17·3	53	+0·3	+0·4	+0·2	+0·30
16·8	98	+0·2	0·0	−0·2	0·00
5·6	85	−0·3	+0·2	+0·2	+0·03
4·0	96	−0·2	0·0	+0·1	−0·03

(*c*) *Body temperature of the alligators.* Two young alligators (*Alligator mississippiensis*) were used for further investigations of the body temperature of reptiles. They were almost of identical size, being 1·08 and 1·10 m. long, respectively weighing 4·77 and 4·33 kg. We have not deemed it necessary to distinguish between them in the record of results.

Table 7 records mean temperature measured under the conditions described in § 2 above. The air temperature and humidity were kept fairly constant during the course of an experiment. To enable body temperature to reach steady value, several hours after placing an animal in the experimental chamber were allowed to elapse before taking readings. From the magnitude of the difference between mean body temperature and mean air temperature, it would seem that the alligator's temperature does not differ significantly from that of the surrounding air over long periods of time, when conditions are maintained relatively stable. Other experiments were carried out to investigate the way in which body temperature lags behind that of the air when it is fluctuating. Fig. 4 is a typical example of such an experiment. The animal was in the experimental chamber over a period of 24 hr. and the air temperature was suddenly changed on two occasions. It reached a maximum value of 32·5° C. and a minimum value of 10·0° C. When the temperature was raised from 22 to 31° C. the body temperature was still 0·5° C. below the air temperature after 3 hr., and when the air temperature was reduced from 32 to 10° C. the alligator still had a body temperature of 0·6° C. higher than the air temperature 9 hr. later.

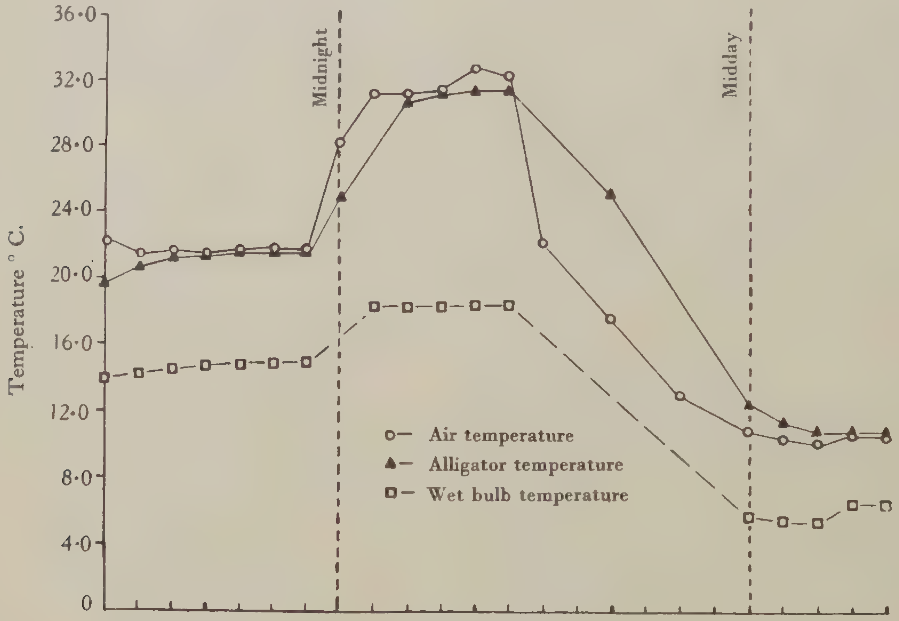

Fig. 4.

Table 7

Exp. no.	Mean air temp. T_1 °C.	R.H. %	Mean alligator temp. T_2 °C.	Range air temp.	Range body temp.	Duration of exp. hr.	$T_2 - T_1$ °C.
1	29·2	33	29·4	±0·6	±0·1	3	+0·2
2	27·8	59	27·5	±0·4	±0·3	4	−0·3
3	25·6	43	26·2	±1·0	±1·0	6	+0·6
4	21·6	78	21·5	±0·3	±0·1	5	+0·1
5	21·1	50	21·1	±0·2	±0·1	4	0·0
6	15·3	83	15·1	±0·1	±0·2	4	−0·2
7	10·3	60	10·7	±0·4	±0·1	4	+0·4
8	7·2	52	7·4	±0·1	±0·1	5	+0·2

5. DISCUSSION

Adaptation of vertebrates to a land habitat necessarily involved a complex set of physiological and anatomical changes which together enabled them to live successfully away from the relatively stable environment of their aquatic ancestors. Structural innovations permitting locomotion on land were of little use without behaviour patterns to ensure the fertilization of eggs in a watery medium, and the advantages of either an impermeable integument or of a calcareous shell during the period of development could not be fully exploited until the tissues were themselves adapted to withstand the effects of high temperature. This adaptation was a necessary precursor of the development of homoeothermy in diurnally active animals. Extensive desiccation would have been the price of stabilizing body temperature at a level lower than that of mammals or birds, and this would have militated against maintenance of stable osmotic pressure of the blood and tissues. Without such osmotic stability, the regulation of a high grade of metabolic and nervous activity such as exists in the mammals and birds would be difficult to conceive.

That such a complex series of changes has taken place is self-evident, but the steps by which they were effected are still obscure from a physiological viewpoint. As has been pointed out previously, Gray (1928) drew attention to the necessity for developing an impermeable integument both for the egg and the adult before extensive conquest of land by vertebrates could proceed. Subsequently, Hall & Root (1930) subjected to experimental enquiry the consequences of the possession of an impermeable integument *vis-à-vis* body temperature. In broad outline, the present investigation supports the main conclusions of Hall & Root, and in particular the thesis that the increase in resistance to desiccation involved a concomitant increase in, if not a predevelopment of, resistance to thermal death at high temperatures. It was, perhaps, unfortunate that circumstances dictated the use of a reptilian species which is by habit cryptozoic. Nevertheless, the thermal death-point of the slow worm is significantly above that of the amphibian species investigated. Although reliable information on the upper thermal death-point of vertebrates is difficult to obtain, the work of other investigators (Cole, 1943) gives us reason to believe that the thermal death-point of typically basking reptiles is considerably above that of the slow worm, and in some species may be as high as 45° C.

In the development of temperature regulatory mechanisms the size of an animal must itself have played a significant part. It has been suggested that the giant reptiles of the Jurassic and Cretaceous may have had a body temperature considerably above the temperature of the air, because of their large bulk and relatively small surface area. This supposition has been discussed by Gunn (1942) who concludes that it is unlikely on the basis of data recorded by Benedict. He suggests that body temperature would follow air temperature sluggishly and fluctuate around a level intermediate between the maximum and minimum experienced during the course of 24 hr. Our own observations on the alligator add weight to this conclusion. The slow worm reaches equilibrium within half an hour, even when temperature changes are sudden and drastic. The body temperature of young alligators of about 4 kg., i.e. one hundred times as heavy as the largest slow worm in our experiments, required about 10 hr. to drop 20° C. when the air temperature was reduced suddenly. The largest extinct reptiles were many hundred times as bulky as our alligators, and it would therefore seem reasonable to suppose that body temperature would fluctuate over a relatively small range. Activity and success could only be assured, however, so long as the mean external temperature remained at a level high enough to permit a rapid movement and the efficient performance of all bodily functions. As the mean external temperature dropped, smaller forms able to take advantage of short periods of high temperature by day and to remain quiescent by night or hibernate during winter would have a better chance of survival. One further consequence of the possession of large body size in reptilian stock would seem to be that a considerable elevation of the metabolic rate would impose upon the animal the need for a cooling mechanism involving copious water loss during periods of high *internal* temperature resulting from previous exposure to high external temperatures.

6. SUMMARY

1. The upper thermal death-point of the slow worm (*Anguis fragilis*) is significantly above that of either the toad (*Bufo bufo*) or the frog (*Rana temporaria*). That of the toad differs only slightly from that of the frog.

2. In response to external variation with respect to temperature and humidity, the body temperature of the toad behaves in a manner similar to that of the frog, being considerably below that of the air at low humidities, though appreciably above that of the wet-bulb thermometer. It is concluded that the toad loses water as freely as the frog.

3. The body temperature of the slow worm and that of the alligator (*A. mississippiensis*) differs only slightly from that of the air under all conditions of *sustained* air temperature and humidity.

4. The body temperature reaction of the alligator is more sluggish than that of the slow worm, presumably owing to the higher thermal capacity of the animal.

5. The significance of these results in relation to the evolution of the terrestrial vertebrates is

discussed. It is suggested that homoeothermic stocks could arise only from reptiles of relatively small size, and that large body size would militate against survival during periods of low *mean* external temperature.

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THE DEVELOPMENT OF THE RAT EYE IN GRAFT

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(*Received* 5 *February* 1946)

(With Plates 8 and 9)

INTRODUCTION

Although many observations have been reported on the development of the embryo amphibian eye when transplanted either into another individual of the same species or into another part of the same animal, experiments on warm-blooded, and particularly on mammalian, material have been much less numerous. Joy (1939) described the differentiation of retina, optic nerve and tapetum nigrum as well as of sclerotic cartilage and choroid tissue from the isolated optic vesicle of 36 hr. chicks transplanted into the coelum of 48 hr. chick hosts and Glees (1941) obtained differentiated retinal, pigment epithelium and lens tissue when the head of a 12-day rabbit embryo was introduced into the pial covering of the cerebral hemisphere of a young rabbit.

In the experiments to be described it was found that when eyes from embryo rats were grafted under the skin of young animals only those parts derived from the mesenchyme were able to survive even a few days whereas good differentiation of all the eye tissues could be obtained if the transplant was made into the rat brain. Such grafts would 'take' even when graft and host belonged to different strains, although in these cases the proportion of failures was higher than when hosts of the same strain were used. An attempt to graft human embryo retina into the rat brain was not very successful.

No 'host reaction' (Loeb, 1930) was observed when transplants were made into the brain, and this finding confirms those of Murphy (1926) and Siebert (1928) that heteroplastic grafts will grow in brain tissue. Murphy believed that a host reaction depends on the presence of a lymphatic system and that the absence of such a system from the brain explains the ease with which grafts of many different tissues can be made into this organ. He also found that embryo tissue showed no defensive reaction to foreign grafts even when these were from a different species, and he explained this result on the same basis since a lymphatic system had not developed in the embryos he used as hosts. That Murphy's views on the all-important part played by the lymphatic system cannot be accepted as they stand is shown by Greene's (1943) success in transplanting alien embryonic tissue into the anterior chamber of the eye and the testicle of adult rabbits and guinea pigs. The host brains used in the present series of experiments were from animals only 2 days old and at this age the rat brain is still not fully developed. Whether the good results obtained were due to the absence of a lymphatic system or to some other property of brain or embryo tissue or to some combination of these factors, it appears that young brains provide a particularly good environment for the development of many different types of graft.

METHOD

The eyes to be grafted were taken from 15-day rat embryos. These were removed from the head under aseptic conditions and as much of the surrounding tissue as possible removed. If necessary they could be kept for several hours in sterile saline.

Under ether anaesthesia a central incision was made in the skin of the host from the neck to the level of the eyes. A semi-circular flap was then cut in the skull over the left hemisphere and a piece of underlying brain tissue removed to make room for the graft, which was then introduced into the hole so formed on the blade of a Graefe knife and the skull flap allowed to drop back into position. Care was taken to avoid cutting the median vein. The skin was then closed over the wound which was painted over with 2% collodion solution. This was found to be a more satisfactory method of closing the wound than stitching. The rat was put into an incubator at 37° C. and not returned to its mother until it had recovered from the anaesthetic. The whole operation was, as far as possible, carried out under aseptic conditions.

The host rats were killed at intervals after the operation varying from 24 hr. to 300 days. When the skin was removed the graft could usually be seen as a pigmented spot just beneath the skull. It often came away with the bone when the brain and skull were separated, but sometimes remained behind embedded in the brain tissue. In the former case the graft was cut off the bone before fixation, in the latter it was fixed together with the surrounding brain tissue.

The specimens were fixed in Zenker's solution and serial paraffin sections made which were stained with haematoxylin and eosin or by the azan method.

In some cases the embryo lens only was grafted and in others the eye was lentectomized before the graft was made.

RESULTS

The mortality among the host rats was very low. Death was usually due to failure to recover from the anaesthetic, but occasionally the young rats were killed or neglected by their mother after the operation. Sepsis in the operation wound was rare. Altogether about 500 operations were performed.

In about 70% of the operations foreign tissue was found in the brain on histological examination. The proportion of 'takes' was less the longer the host was allowed to survive the operation, but healthy eye tissue was found as long as 294 days afterwards when graft and host were of the same strain. The longest survival when the graft was made into a different strain was 167 days.

Often the only eye tissues found when the brain was sectioned were fragments of retina and lens, but in some cases the whole eye was present with all its tissues, those of mesodermal as well as those of ectodermal origin, being easily recognizable and well differentiated. Lachrymal gland, conjunctiva and skin with hair follicles were sometimes found lying outside the bulb in the surrounding brain.

Pl. 8, fig. 1, shows the stage of development reached by the eye at the time of grafting. It will be seen that the development of the lens is nearly complete, but that most other parts of the eye are still in a very immature condition. The retina is composed of two layers only, the optic nerve fibres and a thick layer of undifferentiated cells showing many mitoses along the outer edge. There is no iris or ciliary body and the sclera, choroid and cornea are still in a very rudimentary condition.

Pl. 8, fig. 2, is taken from an eye 13 days after grafting and, since the gestation period of the rat is 21–22 days, is, therefore, equivalent to one from a 6–7-day-old rat (fig. 3). Although the relative dimensions of the eyes in figs. 2 and 3 are not the same and there is some disturbance in structural organization in the grafted eye, it is clear that all the essential structures have differentiated in the graft. There is an easily recognizable ciliary body and iris, the sclera and cornea have developed and the retina has reached the same stage of differentiation as in the normal eye. Lachrymal gland tissue has also developed outside the bulb. Figs. 4 and 5 show the normal eye at 14 days and an eye that was allowed to develop for 21 days in graft so that it is of an equivalent age. Retinal development has proceeded at the normal rate in the grafted eye and the rod layer has appeared.

Pl. 9, fig. 6, shows that after an eye is grafted it does not continue its development undisturbed. This eye was removed 2 days after the graft was made and the picture is typical of the condition found in twenty grafts during the first few days after the operation. Practically the whole contents of the posterior part of the bulb are necrotic, the only healthy tissue left being the pigment epithelium, the extreme periphery of the retina and the anterior epithelium of the lens. This widespread degeneration of the more highly developed parts of the interior of the eyeball is probably due to the removal of its normal circulation when the eye is cut out of the embryo head. Under these conditions only those parts which are in fairly close contact with the cut surface of the host brain (e.g. the pigment epithelium) or those which are still at a relatively early stage of development seem able to survive. After 3–4 days the graft becomes vascularized by ingrowth of vessels from the host brain tissue.

DISCUSSION

It seems that one must assume that the lens and retinal tissue found in those eyes surviving for a week or longer as a graft must develop from the fragments remaining after the degeneration depicted in Pl. 9, fig. 6, has taken its course. The new retinal tissue presumably derived from the surviving ciliary region and the lens fibres from the lens epithelium. No direct evidence was obtained that new retinal tissue is developed from the surviving peripheral ring but, in the absence of indications of another source, this seems the most reasonable assumption. At every stage of development the peripheral part of the retina is less mature than the central (cf. Pl. 8, figs. 3, 4) and mitosis is particularly active in this region. Further, in fig. 4 the increase in amount of retinal tissue and lack of a properly organized structure, shown in the appearance of rosettes in the periphery, suggests that this was a site of rather active growth. Both Ask (1926) and Stone (Stone & Zaur, 1940; Stone & Chace, 1941) reported degeneration of the central retina followed by regeneration in transplanted salamander eyes. The latter states that this regeneration originates in the ciliary region of the retina.

There is no doubt that the production of new lens fibres is due to the activity of the surviving lens epithelium. In several grafts pieces of epithelium became detached and formed fibres on their own away from the main body of the lens, while in others a lens, which appeared on superficial examination to have developed normally, was found to have more than one equator. Pl. 9, fig. 7, shows such a lens. It is from an eye which was allowed to develop in graft for 21 days and is, therefore, equivalent to a normal 14-day lens. By this age the rat lens is fully developed and the anterior part of the one illustrated in fig. 7 is perfectly normal. The epithelium is reduced to a single layer of cells and the equator (*AB*) is easily recognizable. There is, however, another small piece of epithelium at the posterior pole which has produced its own lens fibres, so forming a second equator at *CD*.

The production of lens fibres from isolated pieces of lens epithelium has already been described by several workers. Stone & Sapir (1940) found that a new lens could be produced in lentectomized eyes of anuran, urodele and fish larvae by the implantation of lens fragments so long as these included the equatorial region. They show photographs of multilobed lenses very like that in Pl. 9, fig. 7. They were unable to obtain Wolffian regeneration of the lens in these species. van Deth (1940) described differentiation of lens fibres from epithelium in the embryo chick and Fleisher (1921) claimed that the epithelial cells in his auto- and homografts of guinea-pig lens attempted to regenerate fibres. Riedl (1939) found small free 'lentoids' in eight patients belonging to a family suffering from hereditary perinuclear cataract. In all these individuals the lens had been removed at an early age and Riedl suggests that the lentoids, which were composed of true lens fibres, had developed from fragments of epithelium left behind at the time of the operation.

This regeneration of lens fibres from the epithelial cells cannot be dependent on the presence of other ocular tissue since it occurs when lens epithelium only is transplanted (van Deth) or where the whole lens is removed and grafted. The latter experiment was performed several times by the method described above and in nearly every case one or more multilobed lenses were recovered later. The production of such lenses was taken as indicating that the original fibres had degenerated and been replaced by new ones developed from the surviving epithelial cells.

An attempt was made to discover whether Wolffian regeneration of the lens from the iris epithelium under the influence of the retina (Wolff, 1894) can occur in the rat. In these experiments the lentectomized eye was used for the graft. Although lens tissue was undoubtedly produced in several cases it was impossible to be absolutely certain that no lens epithelium was introduced in the graft. It is extremely difficult to remove the lens from an embryo eye without leaving fragments of its epithelium behind, an experimental complication which has not always been sufficiently recognized in past work on Wolffian regeneration.

The induction of lens tissue from iris epithelium by the retina, first described in Amphibia by Wolff, has been shown by Stone and his colleagues to occur in the salamander (Stone & Chace, 1941; Stone, 1943) but not in some other species of Amphibia or in fish (Stone & Sapir, 1940). Positive results were also obtained by Mouroy (1939), Zalokar (1941) and Dinnean (1942), while Alexander (1942), like Stone, could obtain no regeneration in fish embryo eyes. This type of lens regeneration has also been described in the chick by Barfurth (1906) and, more recently, by van Deth (1940), but does not appear to have been observed in mammals.

SUMMARY

1. Successful grafts were obtained when 15-day embryo rat eyes were transplanted into the brains of 2-day old rats.
2. In the grafts differentiation of the various ocular tissues proceeded at the normal rate.
3. The lens and retina first degenerate and then regenerate, the lens from its epithelium, the retina from its ciliary area.
4. It was not possible to establish whether or not Wolffian regeneration of the lens can occur in the rat.

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EXPLANATION OF PLATES

PLATE 8

Fig. 1. Eye of normal 15-day embryo rat. Zenker. Haematoxylin and eosin. ×64.

Fig. 2. Eye of 15-day embryo rat 13 days after being grafted into the brain of a 2-day-old rat. Equivalent age: 6–7 days. Compare fig. 3. Zenker. Haematoxylin and eosin. ×56.

Fig. 3. Eye of normal 6-day-old rat. Zenker. Haematoxylin and eosin. ×16.

Fig. 4. Eye of normal 14-day-old rat. Zenker. Haematoxylin and eosin. ×16.

PLATE 9

Fig. 5. Eye of 15-day embryo rat 21 days after being grafted into the brain of a 2-day-old rat. Equivalent age: 14 days. Compare fig. 4. Zenker. Haematoxylin and eosin. ×12.

Fig. 6. Eye of 15-day embryo rat 2 days after being grafted into the brain of a 2-day-old rat. All the ocular tissues except the pigment epithelium, lens epithelium and extreme periphery of the retina are necrotic. Zenker. Haematoxylin and eosin. ×56.

Fig. 7. Lens of 15-day embryo rat 21 days after being grafted into the brain of a 2-day-old rat. Note the two equators *AB* and *CD*. Zenker. Haematoxylin and eosin. ×32.

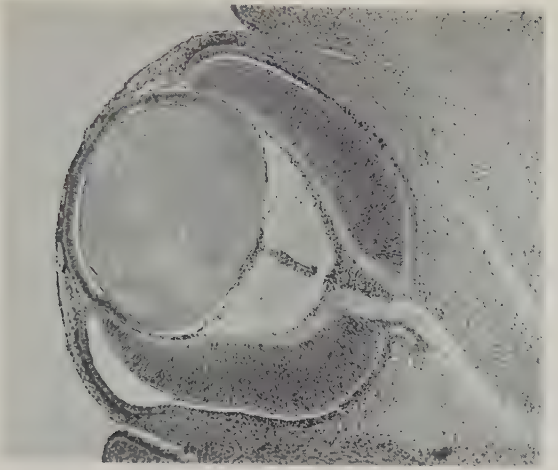


Fig. 1

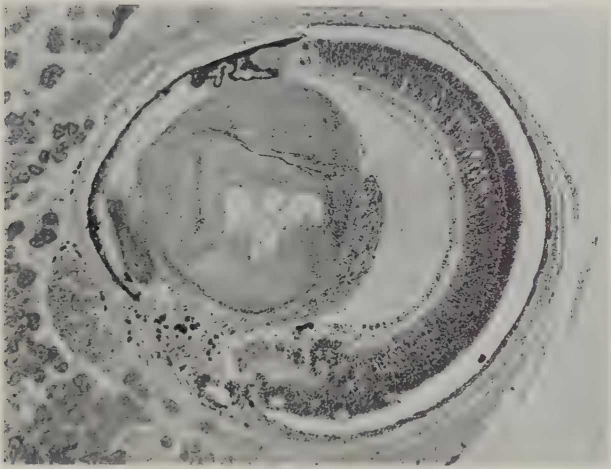


Fig. 2

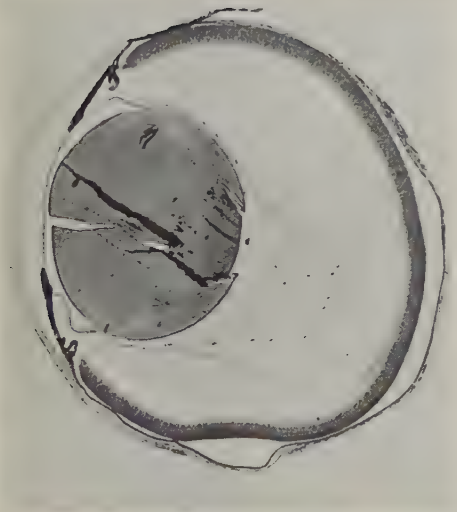


Fig. 3

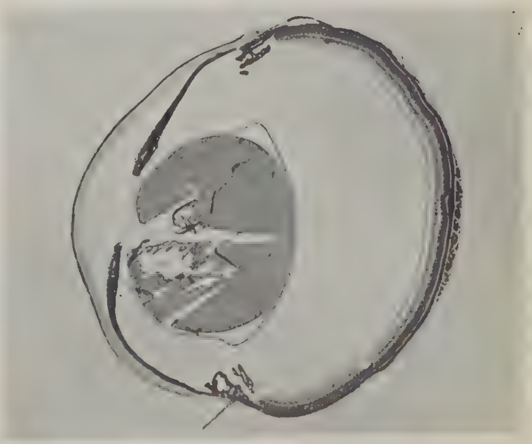
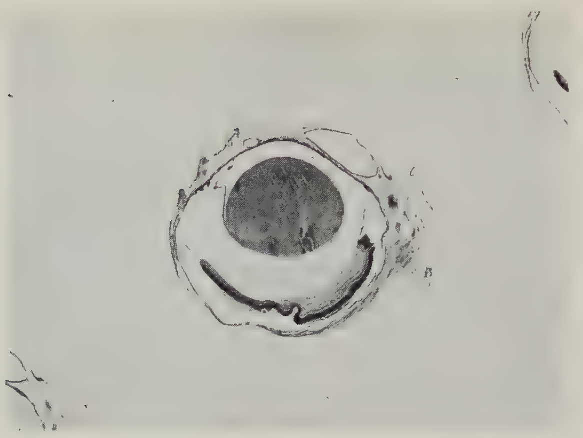


Fig. 4

TANSLEY—THE DEVELOPMENT OF THE RAT EYE IN GRAFT



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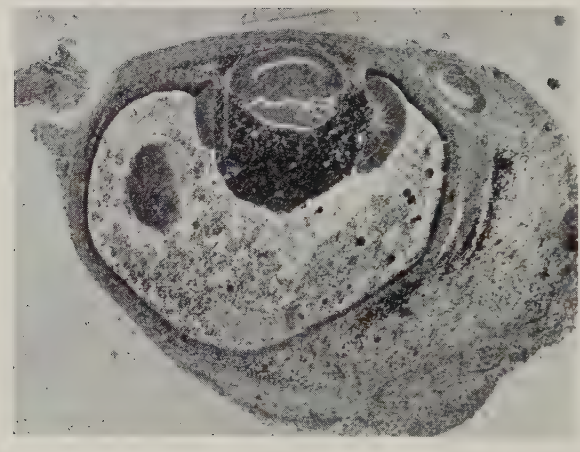
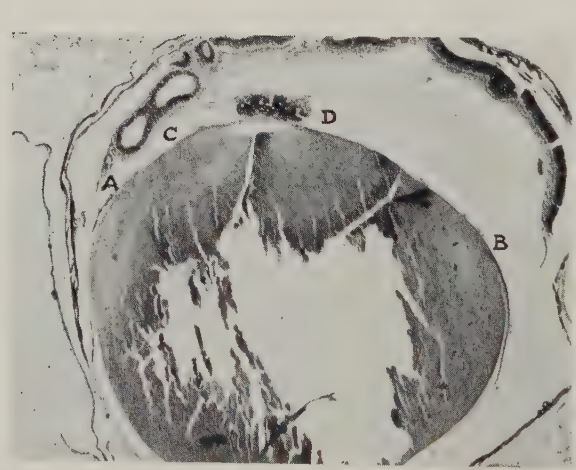


Fig. 6



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